SUBJECT: Risk Assessment for TERA R-19-0001 - **NOT CBI**

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DATE: May 31, 2019

SUMMARY

The Agency received a TSCA Experimental Release Application (TERA) from Synthetic Genomics, Inc. to test one intergeneric eukaryotic algal strain in open ponds. The submission strain for this risk assessment is *Parachlorella* sp. STR26155. The submitter plans to field test this intergeneric algal strain in open miniponds at the Synthetic Genomics, Inc. - California Advanced Algae Facility (CAAF) in Calipatria, CA.

The recipient alga strain is *Parachlorella sp.* STR00012. The submission strain, *Parachlorella* sp. STR26155, contains an intergeneric gene that encodes for a "Turbo" green fluorescent protein (GFP). The family of GFPs and GFP-like proteins, from a wide variety of sources, have been utilized as reporter proteins and are well-characterized in many host systems, with minimal impact to their phenotype.

The submitters selected GFP based on its well- understood characteristics and suitability as a means of identifying the submission strain *Parachlorella* sp. STR26155 in environmental monitoring samples, whether by genetic analysis or microscopy, when cultivated in an open pond research and development (R&D) setting. Preliminary data in the TERA submission (R-19-0001) indicated that the *Parachlorella* sp. STR26155 expressing TurboGFP has no competitive advantages compared to the wild type strain with respect to growth or environmental dispersal.

There is low risk of injury to human health and the environment associated with the small-scale field testing of the intergeneric alga strain *Parachlorella* sp. STR26155. The submission strain does not present concerns for pathogenicity or toxicity to humans. It does not present allergenicity concerns for workers or the general population over that of the recipient algal strain.

The small-scale field testing of the submission strain, *Parachlorella* sp. STR26155, is expected to present low risk to the environment since the introduced genetic material does not impart any competitive growth or dispersal advantages as compared to the recipient strain. Dispersal of the algal cells into the environment is likely since *Parachlorella* is closely related to (and also previously named) the more

widely referenced *Chlorella,* which is an algal species known to be routinely transmitted in air. Members of the genus *Chlorella* are known to survive in marine systems, so it is likely that the submission strain may do so as well. However, the submission strain is not expected to be invasive and outcompete other algae in the environment. Therefore, no adverse environmental effects are expected if the strain does survive in terrestrial and aquatic environments into which it is dispersed.

The horizontal gene transfer of the introduced genetic material, TurboGFP, to other algal species in the environment is expected to be low as *Parachlorella* is not known to readily exchange genetic material horizontally. Vertical transfer of the introduced genetic material through sexual reproduction to other *Parachlorella* species is also expected to be low since *Parachlorella* is thought to be asexual. Thus, there is low concern for transfer of the introduced TurboGFP gene to other algae in the environment. Even though there may be dispersal of the submission strain *Parachlorella* sp. STR26155 into the environment from the proposed small-scale field testing, there is low risk associated with these field tests since the submission strain poses low human health and ecological hazards.

Of note, although the *Parachlorella* genus has not been assessed by EPA, the closely-related *Chlorella* genus, have been approved for two previous TERA applications ([redacted], R-18-0001).

I. INTRODUCTION

EPA has received a TSCA Environmental Release Application (TERA) from Synthetic Genomics, Inc. (SGI) to test one intergeneric eukaryotic algal construct, *Parachlorella* sp. STR26155, in a field trial in open ponds.

The introduced intergeneric DNA gene present in the final construct encodes for TurboGFP. The gene is regulated by the endogenous *ACP1* (Acyl Carrier Protein) promoter and terminator of *Parachlorella*. The expression of TurboGFP will be used by SGI to specifically track the STR26155 strain in open-culture and in the environment.

The selectable marker gene *ble* (for resistance against bleomycin family antibiotics; e.g., zeocin) was also used during intermediate cloning steps, but was removed from the final subject strain via Cre-lox recombinase technology, leaving only a short, non-coding 34bp loxP site as the other intergeneric component. According to the submission, these intergeneric additions to create the subject strain, *Parachlorella* sp. STR26155, resulted in no discernable phenotypic differences relative to the recipient strain STR00012.

The aim of this TERA and the research for which it seeks authorization is, in part, to establish baseline environmental conditions in and around the test facility, and to evaluate and confirm the sufficiency of control and monitoring equipment and techniques developed for this and other similar outdoor R&D programs. This TERA also aims to lay the foundations necessary to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. Gaining insight into how algal strains (top candidates today as well as those to be developed) perform in industrially-relevant settings will inform the design of the technology and ultimately accelerate its development and deployment. It will also reduce the risk of failure that comes with continuing to design a technology without knowing the conditions and constraints it will ultimately face at-scale. The submitter hopes that this effort will contribute to the development of a globally-relevant Safety, Health & Environment package, or "template", for subsequent TERA and Microbial Commercial Activity Notice (MCAN) submissions to EPA and international environmental protection agencies.

II. TAXONOMY AND CHARACTERIZATION

A. Recipient Microorganism

The submitter identifies the parental organism as a wild-type *Parachlorella* sp. (SGI strain designation - STR00010). This strain was isolated from seawater samples collected by SGI near the Hawaiian island of Oahu. *Parachlorella* sp. STR00010 was then subjected to UV mutagenesis to create STR00012, the recipient strain for this TERA, which has higher biomass productivity than STR00010. The taxonomic identity of the recipient, *Parachlorella* sp. STR00012, was verified by SGI using 18S rRNA sequence data. There are currently two accepted species of *Parachlorella*, *P. kessleri*, and *P. beijerinckii*. A third species, *P. hussii* has been proposed Bock et al. (2011) but has uncertain taxonomic status. However, SGI's particular isolate STR00010 could not be assigned to any of these species, so the taxonomic designation is *Parachlorella* sp. These phylogenetic analyses provided by the submitter were confirmed in the Taxonomic Identification Report for R-19-0001 (Strope, 2019).

1. The Genus *Parachlorella*

The *Parachlorella* genus has not been assessed by EPA in other submissions. The closely-related *Chlorella* genus however, has been accessed in two previous TERA applications ([redacted], R-18-0001). Both *Chlorella* and *Parachlorella* are taxonomically classified in the Class Trebouxiophyceae and under the Family Chlorellaceae (Huss et al., 1999). Due to the many similarities in morphology and physiology with microalgae within this class and family, many coccoid green algal (termed 'green ball') groups were previously misclassified under the genus *Chlorella* (Krienitz et al., 2004). As taxonomic identification moved towards more modern molecular phylogenetic approaches (e.g., utilizing sequences of 18S rRNA and ITS2 regions), the genus *Chlorella* was broken up into more distinct genera, one of which being *Parachlorella* (Krienitz et al., 2004). In light of this historical misclassification and recent reclassification within the Chlorellaceae Family, many studies and work done with green microalgae fitting the previous "*Chlorella*" description, are likely applicable to the genus *Parachlorella*.

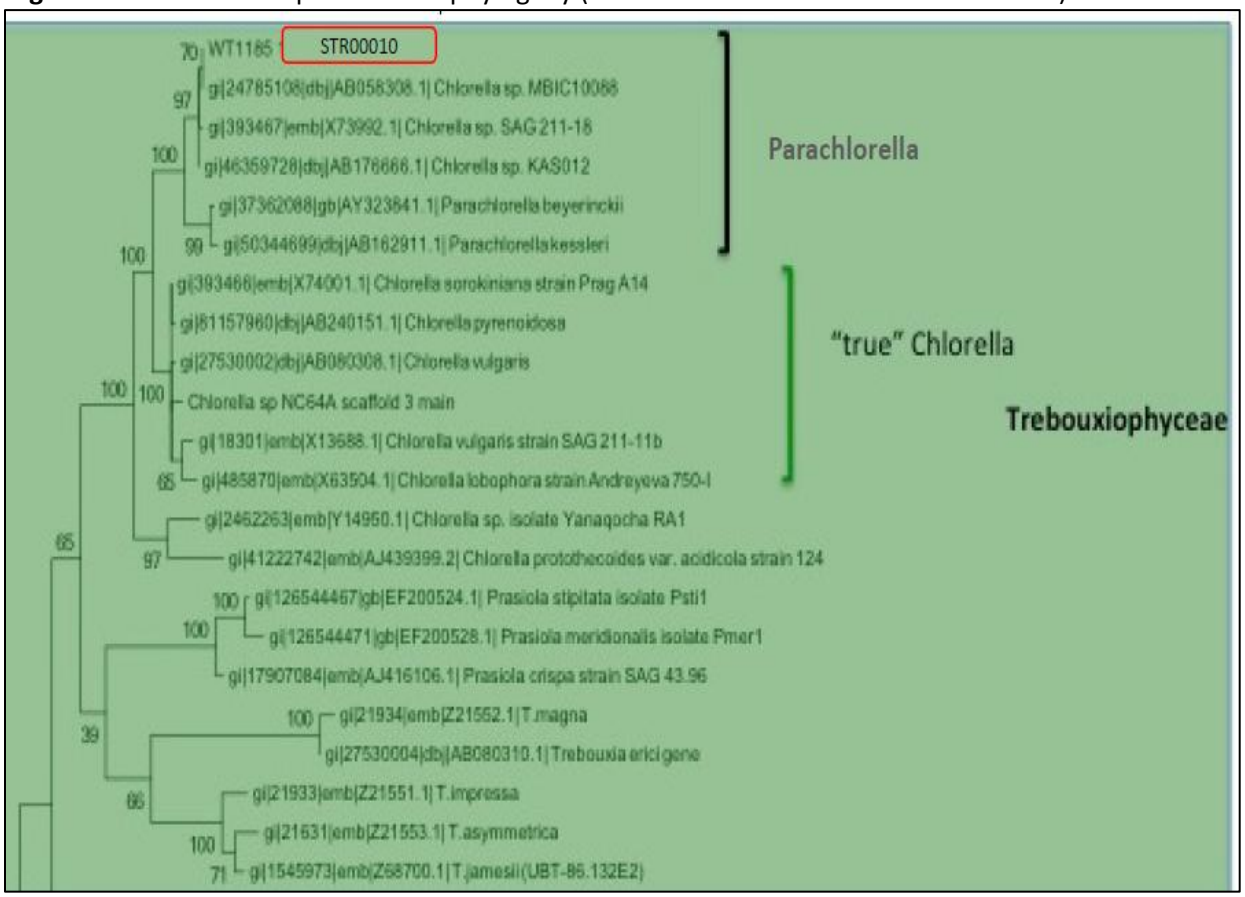
The *Chlorella* genus was first delineated by Beijerinck in 1890. A comprehensive description of the genus *Chlorella* was first addressed by Shihira and Krauss (1965) in response to the lack of a sound taxonomic framework from which to base the identity of over 41 isolates known at the time. In 1976, Kessler identified 77 strains across 12 taxa based on physiological and biochemical properties. Since then the genus has been found to have few useful diagnostically morphological characteristics, making it difficult to identify under a light microscope alone. Only through more rigorous methods (i.e., DNA analysis) can isolates be clearly classified as belonging to a specific species (Bock et al., 2011; Zou et al., 2016). Therefore, a more robust framework, based on polyphasic taxonomic approaches, has been developed to describe well over 100 potentially different *Chlorella* species (Bock et al., 2011; Zou et al., 2016). Based on integrative or polyphasic taxonomy, a new system has been established which differs completely from the traditional artificial system of *Chlorella* and its relatives based on morphology alone. With the introduction of chemotaxonomy to *Chlorella* and other taxa our understanding of the taxonomy of *Chlorella* has changed radically. Based on SSU- and ITS rDNA sequences and light microscopy observations, various publications have demonstrated how the high level of cryptic diversity found within *Chlorella*; and the polyphyletic characters between *Chlorella* and *Dictyosphaerium*, has resulted in numerous taxonomic revisions of these organisms (Zou et al., 2016). For example, Bock et al. (2011) detected six lineages of *Dictyosphaerium*-like strains that are closely related to *Chlorella vulgaris* and described several new species. Krienitz et al. (2015) also attempted to demonstrate that the *Chlorella* species has been widely misclassified when using traditional morphological classification schemes and suggested that only three 'true' spherical species belong to this genus: *Chlorella vulgaris*, *C. lobophora*, and *C. sorokiniana*. Based on biochemical and molecular data, the *Chlorella* genus was even more recently proposed to consist of five "true" Chlorella species (Zou et al., 2016). The number of

Chlorella species appears to have reached ~14 with the inclusion of several former Dictyosphaerium strains (Bock et al., 2011), with suggestions of still others possible ones (Zou et al., 2016).

The submitters provided the following information to support the assignment of *Parachlorella* sp. to their environmental isolate:

> "As part of the process for confirming the correct taxonomic basis for STR00010, we used the nucleotide sequence of the nuclear 18S SSU rRNA, a common phylogenetic marker, to aid in substantiating our strain as belonging to phylum Chlorophyta, class Trebouxiophyceae, order Chlorellales, family Chlorellaceae, genus *Parachlorella*. To place STR00010 in the context of other known *Chlorella* strains, we created a phylogenetic tree based on the analysis of 18S rRNA gene sequences. We selected the 18S rRNA sequences that were previously included in the published analysis of the *Chlorella* NC64A 18S rRNA gene plus the top blast matches to STR00010 rRNA sequence (*Chlorella* strains KAS012, SAG211-18, MBIC10088). The phylogenetic grouping suggests that STR00010 is part of the Parachlorella clade and is divergent from the so-called "true *Chlorella*" clade. While specific phylogenetic relationships continue to be refined, the genus *Parachlorella* was shown to be a sister phylogenetic clade closely related to the "true" spherical *Chlorella*." The resulting phylogenetic tree is shown in Figure 1.

Figure 1. *Parachlorella* sp. STR00010 phylogeny (taken from TERA submission R-19-0001).



2. The species *Parachlorella sp. STR00010*

As previously mentioned, within the genus *Parachlorella*, there are currently two accepted species, *P. beijerinckii* and *P. kessleri* (formerly known as *Chlorella beijerinckii* and *Chlorella kessleri*, respectively). *P. hussii* has been proposed by Bock et al. (2011) and is listed in AlgaeBase (http://www.algaebase.org), but still has an uncertain taxonomic status. The morphological features of these species were described in Buxser (2019), along with closely related organisms (Table 1). These three *Parachlorella* species are

described as solitary or colonial, and sometimes covered by a mucilaginous envelope (Krienitz et al., 2004; Bock et al., 2011). Like *Chlorella* spp., *Parachlorella* spp. are also known to reproduce by autosporulation (typically with 2, 4, or 8 autospores). Other features of *Parachlorella* spp. include a single parietal chloroplast and a broadly ellipsoid pyrenoid, which is covered by starch grains. All *Parachlorella* spp. can be distinguished by substitutions in the 18S rRNA gene sequence, as well as substitutions in the ITS2 region (Krienitz et al., 2004, 2011).

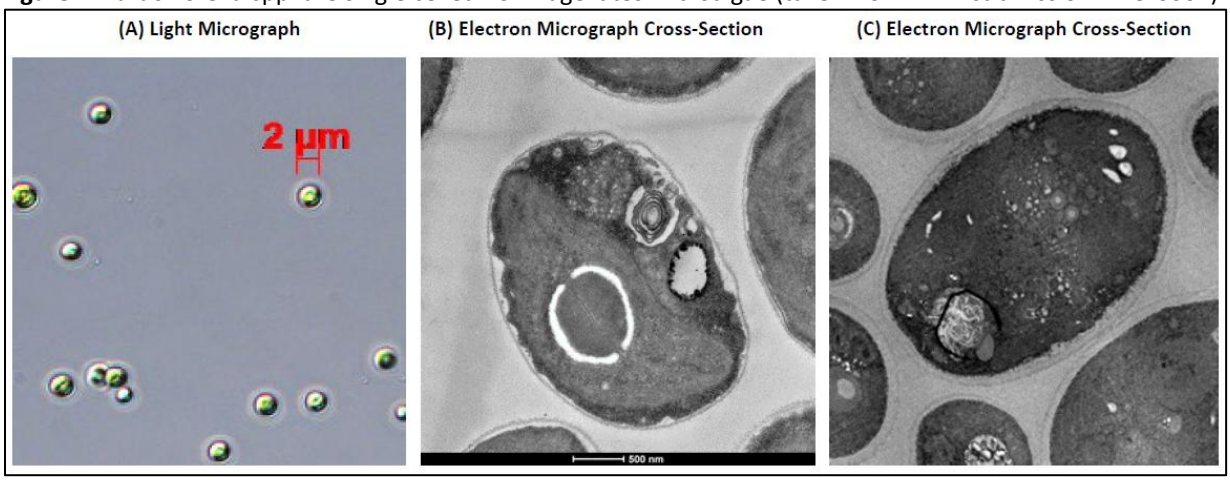
Table 1. Morphological features of *Parachlorella* and genetically-related organisms

Species	Morphology
Chlorella spp.	Cells spherical, subspherical or ellipsoid, single or forming colonies with up to 64 cells, mucilage present or absent. Chloroplast single, parietal, pyrenoid present, surrounded by starch grains. Reproduction by autospores, zoospores lacking. Autospores released through disruption of mother cell wall. Daughter cell can remain attached to remnants of mother cell wall and form colonies with mucilage envelopes. Planktonic, edaphic or endosymbiotic.
Parachlorella spp.	Solitary planktonic or edaphic globose or egg-shaped cells, sometimes with a thin, membranous gelatinous coating; parietal chloroplast with broadly ellipsoidal pyrenoid covered by starch grains; reproduction via 2, 4, 8, or 16 autospores; distinguished from other genera in the family by 18S rRNA and ITS2 nucleic acid sequences.
Parachlorella beijerinckii	As above with cells 2.5-5 x 3-8 µm with a 2-4 µm think gelatinous coat; vegetative cells are spherical or ellipsoidal with 5-8 µm diameter; single pot- or saucer-shaped chloroplast with broadly ellipsoidal pyrenoid covered with 2, 3 or 4 large cup-shaped starch grains; one or two thylakoids traverse the pyrenoid; reproduction by 2, 4 or 8 autospores sized 2.5-3.5 x 3-4.5 µm which were liberated by a broad opening in the mother cell leaving a cup-shaped empty mother cell wall remnant; cells surrounded by amorphous mucilage; electron microscopy revealed a single-layer cell wall; species differentiation by nucleic acid sequencing.
Parachlorella kessleri	In contrast to *P. beijerinckii, P. kessleri* has a mantle-shaped chloroplast and no mucilaginous coat.
Parachlorella hussii	Solitary, planktonic cells with, oval young cells and spherical to slightly oval adult cells 4.5–6.5 (7.5) µm; adult cells are surrounded by a gelatinous coat 1–3 µm thick; a single, parietal, cup-shaped chloroplast and a broadly ellipsoid pyrenoid, which is covered by two starch grains; reproduction by autosporulation with 2, 4 or 8 autospores; species differentiation by nucleic acid sequencing.
Closteriopsis acicularus (in *Parachlorella* clade)	Long needle-shaped with 2 to 6 starch-covered pyrenoids.
Dicloster arcuatus (in *Parachlorella* clade)	Two-celled coenobia with elongated ellipsoidal cells and long pointed apices; a single parietal chloroplast with two pyrenoids.

Table from Buxser (2019)

The parental strain used in this TERA, *Parachlorella* sp. STR00010, along with the derived strains (recipient and subject), were described as being phenotypically and morphologically consistent with a *Parachlorella* assignment. They grow as small (2-3 µm in diameter) unicellular, spherical cells (Figure 2).

Figure 2. *Parachlorella* spp. are single celled non-flagellates microalgae (taken from TERA submission R-19-0001).



B. Donor Microorganisms

The subject strain, *Parachlorella* sp. STR26155, is engineered to express a TurboGFP for monitoring in the environment. TurboGFP is an "improved" variant of the ppluGFP2 originally isolated from the copepod *Pontellina plumata* (phylum Arthropoda; subphylum Crustacea; class Maxillopoda; subclass Copepoda; order Calanoida; family Pontellidae) (Shagin et al., 2004). This Copepoda specimen was specifically found in samples collected in the Gulf Stream, 120 miles east of Charleston, S.C. (Shagin et al., 2004).

1. TurboGFP

GFPs from various sources have been utilized as reporter proteins and are well-characterized in many host systems with minimal impact to their phenotype (Shagin et al., 2004).

The original "wild-type" version of TurboGFP, ppluGFP2, was identified and cloned along with other GFP and GFP-like proteins from Copepoda (Shagin et al., 2004). The name "TurboGFP" was later termed by Evdokimov et al. (2006) after they created an improved variant of ppluGFP2, where the maturation time was decreased, along with its tendency to aggregate *in vitro*. Evrogen (Evrogen Joint Stock Company, Moscow, Russia) then utilized a previous codon-optimization strategy developed by Haas et al. (1996) to allow for overexpression in mammalian systems. However, it still retains successful expression in many other systems expanding its usage as a reporter protein. This specific TurboGFP was purchased from Evrogen by SGI and used in STR26155.

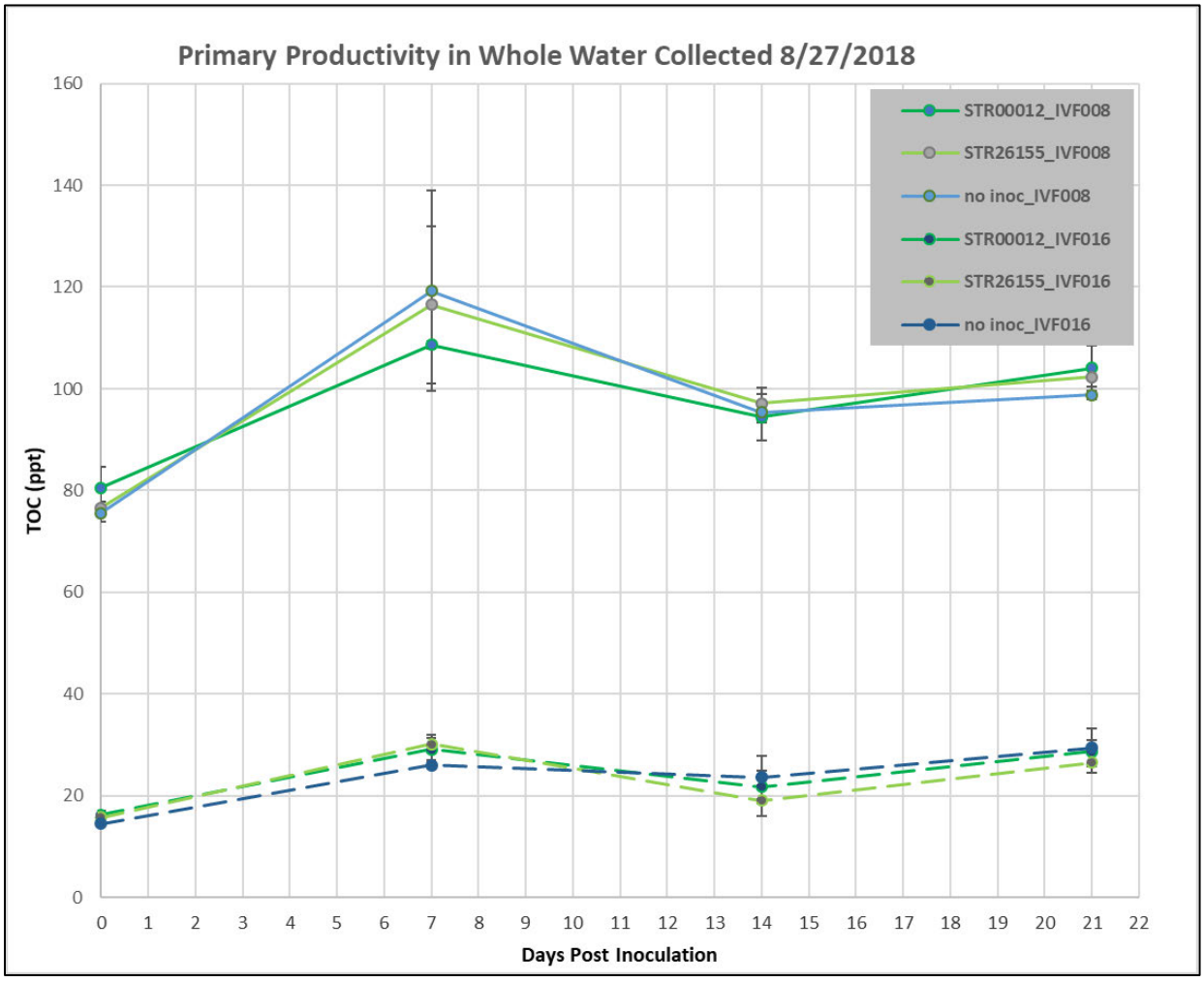
2. loxP site

A single loxP site remains in the genome of the subject microorganism *Parachlorella* sp. STR26155. The loxP site (34 bp sequence originally from bacteriophage P1) was part of the Cre-Lox system, a cloning strategy whereby Cre recombinase results in the recombining of the loxP sites with the excision of DNA contained between the two loxP sites. This system was used by the submitters to remove a selectable marker gene (*ble*). This remaining loxP sequence is non-coding and serves no function in the final subject strain *Parachlorella* sp. STR26155.

C. Submission Microorganism

The addition of TurboGFP is expected and was shown by the submitter to have no discernable phenotypic differences in the subject strain STR26155 relative to the recipient strain STR00012. Various growth tests were performed to ensure that the subject strain has no greater propensity to impact primary productivity than the recipient strain. This was shown to be true and presented in Figure 3 below.

Figure 3. Whole-culture primary productivity (as Total Organic Carbon) measurements for waters spiked with recipient Parachlorella STR00012 (dark green), subject strain STR26155 (light green), and negative controls (blue) in unfiltered local waters. Waters collected from two sites, Salton Sea (IVF008, solid lines) and IID managed marsh (IVF016, dotted lines) on 8/27/2018. (Figure F8 from R-19-0001)



The submitter also conducted a detailed photophysiological comparison of the recipient and subject strains and the data are shown in Table 2.

> "Biological duplicate cultures were acclimated to low light conditions prior to photo-phenotyping. Measurements were made of the maximum quantum yield of photochemistry in PSII (as Fv/Fm), functional absorption cross-section of PSII, light-saturated electron transport rate, Pmax by 14C incorporation, as well as chlorophyll (Chl) a and Chl b content of cells."

They concluded that there was no significant difference between recipient and subject strains as for all

measures the differences between strains were less than the error of the measurement (CVs typically less than 5%). This experiment verified the absence of any photophysiological differences between the strains.

Table 2. Photophysiological characterization and comparison of recipient and subject strains. Errors are given in parentheses. (Table A1 from R-19-0001)

Strain#	FIRe (JTS-10)			^{14}C	Chlorophyll	
	F_V/F_M	σ_{PSII} (Å^2, @ 530 nm)	$1/\tau'_{Qa}$ (s^{-1})	P_{max} (nmol ^{14}C/ mg Chl/ hour)	(Chl a/TOC, %)	(Chl b/TOC, %)
STR00012	0.706 (0.010)	98 (2)	63 (2)	240 (1)	4 (0)	1 (0)
STR26155	0.708 (0.010)	101 (1)	64 (4)	238 (5)	4 (0)	1 (0)

JTS-10 parameters	Description
Fv/Fm	Maximum quantum yield of photochemistry in PSII, measured in a dark-adapted state (dimensionless). This parameter characterizes the efficiency of primary photosynthetic reactions.
σ_{PSII}	Functional absorption cross section of PSII (Å^2) in a dark-adapted state. The parameter is the product of the optical absorption cross section of PSII (i.e., the physical size of the PSII unit) and the quantum yield of photochemistry in PSII.
$1/\tau'_{Qa}$	Light saturated rate of electron transport on the acceptor side of photosystem II. This parameter indicates efficiency of linear photosynthetic electron transport

III. ECOLOGICAL INTERACTIONS OF ALGAE IN THE ENVIRONMENT

The interactions of algae in aquatic and terrestrial environments and their role in aquatic food webs were discussed in a previous risk assessment for an algal submission by McClung (2013).

A. Aquatic Ecosystems

A number of factors affect the rise and fall of algal populations in the aquatic environment including the physical factors of light, temperature, weather, water movements, flotation, the chemical nutrient status of nitrogen, phosphorus, silicon, calcium, magnesium, potassium, sulfate, chloride, iron, manganese, and other trace elements, and organic matter (Ikawa, 2004). There are a number of biological factors as well including the presence of resting stages, predation, and parasitism. The polyunsaturated fatty acids produced by algae can affect algal growth. In addition, a number of biological substances are known to be produced by algae that inhibit the growth of other algal or of zooplankton grazers, as shown by Pratt (1944; Pratt et al., 1945). Likewise, it has been shown that some algae detect "infochemical" signals from grazers and can change their morphology accordingly to try to avert predation (Lass and Spaak, 2003). Food webs in water bodies are complex and dynamic and have been shown to vary from season to season and with other perturbations of the water body. e.g., eutrophication (Lindeman, 1942; Martinez, 1991).

In terms of symbiosis, *P. kessleri* along with six other algal strains previously classified as "*Chlorella*" were able to form stable symbiotic relationships with *Hydra viridis* (freshwater polyp) (Kessler et al.,

1998). Competition studies have also indicated that *Parachlorella* spp. can survive in the presence of toxic cyanobacteria (Peng et al., 2011). The submitters validated this with their own invasion/competition type experiments using both the recipient and subject strains, showing their ability to "persist in the face of competition from indigenous species".

Algae and cyanobacteria are the basis of the food web in both freshwater and marine aquatic ecosystems. The phytoplankton community of a typical north-temperate lake has been shown to consist of up to several hundred algal species that co-exist (Kalff and Knoechel, 1978). Phytoplankton diversity is influenced not only by the different ecological niches within a water body (e.g., benthic vs. pelagic regions), but also by a number of temporal and spatial variations in factors such as nutrient supply, temperature, dissolved oxygen, predation, and parasitism (Wehr and Sheath, 2003; Townsend et al., 1998). Nutrient supply and herbivory are thought to be the most important parameters affecting diversity changes over time. According to Wehr and Sheath (2003), the phytoplankton species composition in lake food web ecosystems is important because the 'functional properties of algal assemblages vary strongly with species composition'. Different taxa are important because features that are sometimes used to classify various species such as photosynthetic pigments, storage products, motility, reproduction, cell ultrastructure, and even DNA sequence have functional importance. For example, nitrogen fixation ability is of great functional importance but is restricted to a limited number of cyanobacteria. Also, photosynthetic pigment production is important, for instance with the red accessory pigment phycoerythrin which has an absorption maximum of 540-560 nm. The presence of this pigment broadens the photosynthetic capacity of an ecosystem by facilitating growth at greater depths (Goodwin, 1974). Autotrophic picoplankton have a strong competitive advantage under phosphorus-limiting conditions (Suttle et al., 1988; Wehr, 1989).

Diversity in the size fractions of phytoplankton is an important aspect of algal communities and thus food webs. For planktonic food webs, cyanobacteria have a dominant role in aquatic productivity. It is these smaller autotrophs that provide excreted dissolved organic compounds that provide substrates for heterotrophic bacterial growth. In addition, cyanobacteria are directly grazed by protozoa (microflagellates and ciliates). This microbially-based food web in which the major portion of autotrophic production occurs is important to the marine food webs. The microbial food web consists of those organisms that are < 1000 µm, and in freshwater benthic ecosystems consists of (presented by increasing size fraction) cyanobacteria and bacteria, followed by microflagellates, diatoms and green algae, which are then consumed by ciliates, rotifers, copepods, oligochaetes, nematodes, and then invertebrate macrofauna followed by the larger vertebrates (Bott, 1996). A complex microbial food web has bacteria and algae at the lowest trophic level, which are then consumed by protozoa and meiofauna. Meiofauna are organisms in the size range of approximately 50 - 1000 µm and includes large ciliates and metazoan (e.g., rotifers, copepods, and oligochaetes).

An important link between microbial food webs and classical food webs are with the autotrophic picoplankton (> 0.2 - 2 µm). These cyanobacteria are grazed mainly by micro-zooplankton (ciliates, flagellates) rather than by cladocerans or copepods (Pernthaler et al., 1996; Hadas et al., 1998). Size affects the sinking rate with smaller planktonic species sinking more slowly. Thus, the smaller species remain more prevalent in the euphotic zone.

B. Terrestrial Ecosystems

Algae occur in nearly all terrestrial environments on earth, including desert soil crusts, and are invariably encountered on and beneath soil surfaces (Metting, 1981). Acceptance of algae as bona fide soil microorganisms occurred late in the 19th century when it was recognized that certain groups were restricted to soil, including some *Chlorella* species (Shihira & Krauss, 1965; Kessler, 1976). Over 38 prokaryotic genera and 147 eukaryotic genera have been identified as terrestrial species, the majority of

which are truly edaphic. As expected, solar radiation, water, and temperature are the most important abiotic factors controlling their distribution, metabolism, and life histories (Metting, 1981). Biotic interactions are also important, but much less well understood. Algae play an important role in primary and secondary plant community succession by acting as an integral part of ecosystem. Algal communities living in soil have the principal function of primary productivity, nitrogen fixation, and stabilization of aggregates, i.e., prevention of soil erosion (Metting, 1981). Algae concentrations in soils are typically found to be between 10^3 and 10^4 cells/gram dry soil but have been reported as high as 10^8 (Metting, 1981).

IV. DISPERSAL OF ALGAE IN THE ENVIRONMENT

As reviewed by Tesson et al. (2016), microalgae have been reported across a wide range of ecosystems, covering almost all latitudes from tropical to polar regions. Due to their relatively small size (few to 500um), microalgae are dispersed by water, air, and various biotic vectors (e.g., humans and animals) (Kristiansen, 1996a; Tesson et al., 2016). These mechanisms and organisms of dispersal were discussed in a previous algal risk assessment by McClung (2017).

A. Dispersal by Water

Passive dispersal of algae by water can occur wherever there is running water between connected water bodies. A study by Atkinson (1988) found that the colonization of a newly constructed reservoir was from the inflow, and it took several years before there was the appearance of organisms different from those found in the catchment area. Heavy precipitation and flooding can result in algal dispersal by connecting water bodies that are usually isolated. Algal dispersal by water is likely more important in wetter environments that in arid regions.

B. Dispersal by Aerosols

Air currents are an important dispersal mechanism for algae, and it is thought that algae have spread throughout the globe as aerosols. As early as 1844 Ehrenberg recognized the presence of airborne algae in dust samples collected 300 km off the nearest coast by Darwin in 1939 on the H.M.S. Beagle (Kristiansen, 1996b).

According to a review article by Sharma et al. (2007), ”In general, bioaerosols range from 0.02 to 100 µm in diameter and follow the same physical rule as any particle of a similar aerodynamic diameter. They disperse via air movements and settle according to the settling velocity, available impaction, surface, and climatic factors prevailing in the area (Burge and Rogers, 2000). Air movements within a laminar boundary layer surrounding the source usually release such particles. Many of the particles remain in the layer and eventually settle near the source (<100 m), while some are carried aloft with turbulence and transported by the wind over a long distance. The processes responsible for the release and atomization of bioaerosols from natural sources are as follows:

1. Sweeping of the surface or rubbing together of adjacent surfaces by wind and gusts dislodges the bioparticles from the surface. Dried algae caught by the wind are carried away like dust particles (Grönblad, 1933; Folger, 1970).

2. Formation of oceanographic aerosols by wave action and the bursting of bubbles at the water-air interface (Woodcock, 1948; Stevenson and Collier, 1962; Maynard, 1968; Schlichting, 1974). Fragments of scums and foams with algal contents along the shoreline of water bodies can be picked up by the wind and carried aloft (Maynard, 1968).

3. During heavy rainfall, algae are splashed up by raindrops and can be entrained into the atmospheric air by thermal winds (Burge and Rogers, 2000).

4. Storm activity over land and sea where great turbulence is experienced.

5. Human activities, such as agricultural practices, construction and maintenance practices, sewage treatment plants (Mahoney, 1968, as cited in Sharma et al., 2007), garbage dumping, highway traffic, and to a limited extent weapons testing and spacecraft launching, can result in the atomization of constituting algae (Schlichting, 1974; Kring, 2000).

6. Atomization of aerosols to a low height also occurs when surface water containing blooms is used for irrigation and recreational activities like boating, jet skiing, and so forth (Benson et al., 2005)".

Sharma et al. (2007) also stated, based on the result of earlier publications, that green algae, cyanobacteria, diatoms, and tribophytes comprised most of the aero-algae flora. Cyanobacteria dominate the aero-algae flora of tropical regions whereas chlorophytes (green algae) dominate in the temperate regions.

Brown (1964) conducted studies on airborne algae using agar petri dishes suspended in stationary locations in Texas, and impaction studies of algae onto agar petri dishes collected from moving automobiles in 14 states. He also collected samples from an airplane. The impaction from the moving automobiles and planes yielded the greater numbers and diversity of algae. For example, the agar plates held from a moving car in Pennsylvania yielded 140 algal impactions composed of approximately 25 different genera of algae. A 10-second exposure obtained from a moving car sampling a local dust cloud resulting from plowing of a field recorded 5000 algal compactions, of which 4500 were chlorophycean or xanthophycean. *Chlorella* was one of the algal genera found. The algal content of dust was found to be quite high at > 3000 cells per m^3. The author concluded that soil is the predominant source of airborne algae.

Schlichting (1969) conducted studies on airborne algae in Michigan and Texas using Millipore filters and bubblers containing soil-water extracts at heights of 6, 15, 30, 75, and 150 feet from the ground. Also, aerial sampling of maritime algae was made from a ship 100 miles off the coast of North Carolina. Over an eight-year period, the number of algae collected never exceeded 8 cells/ft^3. He then estimated that a person at rest would inhale 240 algal cells per hour which would result in an inhalation exposure of approximately 2880 cells/day. Higher algae numbers were found in the Texas samples from dust than those from water environments. In a summary of the existing literature on algae found in aerosols, viable cells of *Chlorella* were sampled directly from the air in these states and in Holland and Taiwan. Species of *Chlorella* found included *C. ellipsoidea, C. pyrenoidosa, C. vulgaris*, and *Chlorella* sp. (Schlichting, 1969).

The diversity and abundance of airborne green algae and cyanobacteria on monuments and stone art works in the Mediterranean Basin was studied by Macedo et al. (2009). Airborne *Chlorella, Stichococcus,* and *Chlorococcum* were the three most frequently encountered chlorophyte.

The diversity of aero-algae in a Mediterranean river-reservoir system was found to be high (Chrisostomou et al., 2009). They found that nanoplanktonic algae comprised the majority (46.4%) of the aero-algae flora with *Chlorella* being the predominant aero-alga. Three of the most frequently isolated nanoplanktonic airborne algae were *Chlorella vulgaris, Didymocystis bicellularis,* and *Scenedesmus obliquus.* The authors suggested that these vegetative cells have a protective external coating that allows them to resist desiccation in bioaerosols for short distances.

Genitsaris et al. (2011) did a comprehensive review of studies in the published literature on airborne algae. They summarized that the most frequently occurring algae isolated from aerosols were *Chlorella, Scenedesmus, Chlorococcum,* and *Klebsormidium,* and the cyanobacterium *Lyngbya.* These were found in more than 40% of the sites that had been sampled by various researchers in their aero-algae studies.

In aquatic habitats, microorganisms are known to be concentrated in the surface films and in foams on the water surfaces (Maynard, 1968). Schlichting (1974) conducted studies on the ejection of microorganisms into the air with bursting bubbles. He found that bubbling air through a bacterial culture resulted in 2,000 times more bacteria in the bubble jet droplets. Microorganisms in the range of 0.3 to 30 µm in diameter can be carried in atmospheric water droplets (Woodcock, 1948, as cited by Schlichting, 1974).

Airborne algae are subject to desiccation stress and ultraviolet light exposure (Sharma et al., 2007). Desiccation, the equilibration of an organism to the relative humidity of the surrounding atmosphere, is an intensive stress that typically, most phototrophic organisms cannot survive (Holzinger and Karsten, 2013). However, there are studies that suggest that some algae can survive desiccation stress (Evans, 1958, 1959; Schlichting, 1961). A comprehensive list of algae capable of surviving desiccation was published in 1972 by Davis. Parker et al. (1969) reported that various cyanobacteria and green algae survived desiccation as viable algae were found in decades-old air-dried soil samples. This is in contrast to Schlichting (1960) who reported survival of only four hours with desiccation stress. Ehresmann and Hatch (1975) studied the effect of relative humidity (RH) on the survival of the unicellular eukaryotic alga *Nannochloropsis atomus* and the prokaryotic alga *Synechococcus* sp. Viable cells of the latter species could be recovered at all the RHs tested (19, 40, 60, 80, and 100%). However, there was a progressive decrease in the number of viable *Synechococcus* cells with lower RHs. There was a stable survival at RH 92% and above. The results with the eukaryotic green alga were very different. No viable cells of *N. atomus* were recovered below 92% relative humidity. In an earlier study Schlichting (1961) found that algae remained viable under a wide range of environmental conditions including RHs of 28-98%. The stress associated with atomization of the algae was responsible for rapid decrease in viability. So perhaps, the gradual air-drying of soil samples as in Parker et al. (1969) did not result in death of the microorganisms.

Recent work by Szyjka et al. (2017) has demonstrated that cultivation of genetically engineered (GE) algae in outdoor ponds can lead to the aerosol release of these organisms. Their data show that algae grown in ponds can travel and be detected in trap buckets as a function of distance and wind direction. Using qPCR to detect both wildtype and the GE strain showed detectable levels in all traps at distances from 5-50 meters away. However, neither strain was able to outcompete local or airborne algae taxa in either the trap buckets or in experiments conducted using local eutrophic and oligotrophic lake water containing local taxa. Their research also showed that airborne algae have high diversity (species detected using ITS2 primers) and can invade any available waters, including members of the species being tested. Aerophilous algae, such as *Chlorella*, can and will travel both short and possibly long distances when grown in open ponds. Thus, it is important to know an alga's ability to survive, establish, and persist in the receiving environment. Additionally, knowledge of the potential for horizontal gene transfer of the introduced genetic material is important as the same species as the recipient alga or close relatives of the species may be found in the surrounding environment, in both terrestrial and aquatic environments.

C. Dispersal by Aquatic and Terrestrial Organisms

Aquatic and terrestrial organisms are also responsible for algal dispersal (Kristiansen, 1996b). Even fish can act as vectors. For example, numerous species of plankton algae including cyanobacteria, green algae, and diatoms have been found to pass undamaged through the digestive track of the plankton-

eating gizzard shad (Velasques, 1939 as cited by Kristiansen, 1996b). Insects such as beetles have been found to carry viable algae in their digestive tract (Parsons et al., 1966, as cited by Kristiansen, 1996b), and thus, their fecal pellets can distribute algae to new water bodies. Milliger and Schlichting (1968) found 20 species of green algae in the intestinal tract of beetles. Algae dispersal by beetles is a likely mechanism for small water bodies for short distances (Kristiansen, 1996b). Other insects can disperse algae to various water bodies. Revill et al. (1967) found that with four species of aquatic Diptera (craneflies and midges), 21 different genera of algae were found on the collected insects. Likewise, Sides (1968) found that the mud dauber wasp was capable of carrying algae and protozoa as nine and four genera, respectively, were isolated from aseptically collected insects. Parsons et al. (1996, as cited by Kristiansen, 1996b) reported the presence of 20 genera of viable blue-green algae (currently cyanobacteria), green algae, and euglenoids in and on dragonflies and damselflies. Dragonflies are thought to be able to transport algae possibly long distances (Maguire, 1963).

Water-living mammals and other mammals such as mink, muskrats, and raccoons can transport viable algae on their fur and sometimes in their intestinal tracts. Human activities (e.g., boating, fishing, hunting) can also transport algae between water bodies. For instance, the use of felt-soled wading boots has been banned in a number of states as they have been shown to transport non-native larvae, spores, and algae between water bodies. In Vermont, the felt-soled wading boots are believed to have spread didymo, a slimy alga also called rock snot, to various rivers throughout the state. This alga forms dense mats that blanket the bottom of the stream like a shag carpet, changing pristine trout streams to a green, yucky mess, according to a fisheries biologist with the state Fish and Wildlife Department (http://usatoday30.usatoday.com/news/nation/environment/2011-04-28-rock-snot-felt-sole-wader-ban_n.htm).

D. Dispersal by Birds

Water birds are one of the most important vectors for algae dispersal as they can transport live algae on their feet and feathers and sometimes internally in their bills or in their digestive tract (Kristiansen, 1996b). Water birds such as seagulls have been shown to transport algae, particularly aquatic desmids, in wet mud on their feet for long distances (Strøm, 1926). Desiccation is of course of great importance with the viability of live algae transported on the feathers or feet of birds. Algae carried internally in the digestive tract are not subject to desiccation stress.

Migratory birds have a significant role in the transport of algae for long distances (Kristiansen, 1996b). Proctor (1959) studied the carriage of algae in the intestinal tract of numerous migratory bird species obtained from playa lakes in Texas and Oklahoma. A number of freshwater algae species were found in the alimentary canal of 25 different migratory birds. Algae were found in the lower digestive tract of the pied-bill grebe, the green-winged teal, the blue-winged teal, the shoveler, the American coot, the killdeer, the dowitcher, the American avocet, the Wilson's phalarope, and the belted kingfisher. Since many species of blue-green algae (currently cyanobacteria) and green algae do not have spores or specialized resting structures, the algae were assumed to have been transported as vegetative cells. Based upon the rate of movement of the algae through the alimentary tract and the flying speed of some common migratory birds, Proctor (1959) suggested that algae could be easily transferred between lakes 100 - 150 miles apart, with much greater distances possible with cells or colonies in the caecum of the birds.

Schlichting (1960) also investigated the transport of algae on and in various waterfowl. He measured the carriage of chlorophyta (green algae), cyanophyta (blue-green algae), chrysophyta (golden algae), euglenophyta, bacteria, fungi, protozoa, and rotifers and on the feet and feathers, and in the bill and gullet, as well as in the fecal matter of 105 birds representing the following 16 species of waterfowl: black duck (*Anas rubripes*), blue goose (*Chen caerulescens*), buffle-head duck (*Bucephala albeola*),

Canada goose (*Branta canadensis*), coot (*Fulica americana*), Eastern belted kingfisher (*Megoceryle alcyon*), gadwall *(Anas strepera*), goldeneye (*Glaucinetta clangula americana*), green-winged teal (*Anas carolinensis*), mallard (*Anas platyrhynchos*), redhead duck (*Aythya americana*), ring billed gull (*Larus delawarensis*), ruddy duck (*Oxvura jamaicensis*), spotted sandpiper (*Actitis macularia*), common snipe (*Capella galinago*), and wood duck (*Aix sponsa*).

The field collection experiments demonstrated that the water birds retained viable forms of algae and protozoa both externally and internally. For those organisms carried externally on the feet and feathers, the birds exposed to the air for less than four hours carried a great variety of organisms. Those exposed to air for longer periods of time had fewer viable organisms. With eight hours of exposure to air, there were some organisms on the feet of birds, but a greater variety was found to be carried in the bills. The birds exposed to the air longer than eight hours yielded very few organisms. The contents from the gullets sampled produced good algal growth in culture, whereas only a few of the 163 fecal samples contained viable algae or other organisms. Viable organisms found on the waterfowl consisted of 86 species from the feet, 25 species from the feathers, 25 species from the bills, 14 species from the gullets, and 12 organisms from the fecal material.

The following species of green algae were found on the feet of the waterfowl: *Ankistrodesmus braunii, A. convolutus, A. falcatus, Arachnochloris-like cells, Arthrospira gomotiana, A. jenneri, Chlamydomonas globosa, C. mucicola, C. pseudopertyi, C.* sp.*, Chlorococcum* sp.*, Chlorella ellipsoidea, C. vulgaris, Chlorella* sp.*, Closteriopsis-like cells, Dactylococcopsis acicularis, Franceia* sp.*, Glenodinium* sp.*, Gloeocystis gigas, Mougeotia* sp.*, Nannochloris bacillaris, Oedogonium* sp.*, Oocystis rorgei, Palmodictyon* sp.*, Protococcus* sp.*, Rhabdoderma irregulare, Rhizoclonium fontanum, Scenedesmus abundans, S. dimorphus. S. quadricauda, Scenedesmus* sp.*, Sphaerocystis, Schroeteri, Tetraedron minimum, T. sisconsinense, Tetraedron* sp.*,* and *Ulothrix* sp. The cyanobacteria found on the feet included the following species: *Anabaena affinis, Aphanocapsa* sp.*, Aphanothece castagnei, A. nidulans, Chroococcus dispersus, C. minutus, Gloeocapsa* sp.*, Gloeothece linearis, Lyngbya attenuata. L. limnetica, L.* sp.*, Microcystis aeruginosa, Nostoc* sp.(?), *Oscillatoria angustissima, O. limnetica, O. subbrevis, O. tenuis, O. terebriformis, Oscillatoria* sp.*, Pelo-gloea bacillifera, Phormidium mucicola, P. tenue, Phormidium* sp.*, Plectonema nostocorum,* and *Synechococcus aeruginosus.*

Although much fewer numbers of green algae, cyanobacteria, golden algae, euglenoids, protozoa, and fungi were found on the feathers and bills, *Chlorella sp.* was found in both. It was also speculated by Schlichting (1960) that some microalgae, specifically *Chlorella*, may become embedded in the matrix of larger taxa, such as *Gloeocystis*, and be able to be transported away not only far but protected for greater periods of time.

V. HISTORY OF USE

As stated previously, although the *Parachlorella* genus has not been assessed by EPA, the closely-related *Chlorella* genus however, has been accessed in two previous TERA applications ([redacted], R-18-0001). Both *Chlorella* and *Parachlorella* are taxonomically classified in the Class Trebouxiophyceae and under the Family Chlorellaceae (Huss et al., 1999).

Chlorella (which used to include members now identified as *Parachlorella*) has a long history of research and experimentation, as it is a genus that can be found in marine, freshwater and edaphic habitats; making it one of the most ubiquitous and famous microalgal genus worldwide. Much of what was first discovered about the fundamentals of photosynthesis and inorganic nutrition came from experiments using *Chlorella* (Shihira and Krauss, 1965).

Various *Chlorella* (*including Parachlorella*) species, have been extensively researched for their

application in feed, food, nutritional, cosmetic, pharmaceutical and biofuels (Kang et al., 2004). *Chlorella* is not only a good genus for basic research but also a powerful superfood and has been proposed as a significant player in the development of second-generation biofuels and medical treatments (Kumar et al., 2016; Pienkos and Darzins, 2009). The genus *Parachlorella* specifically has been used in aquaculture as food for several shrimp species, including shrimp that is ultimately sold for human consumption (Ueno et al., 2016).

VI. CURRENT USE AND FUTURE USES

Per the TERA (R-19-0001), SGI and ExxonMobil's ultimate goal is to develop renewable, sustainable, low-carbon, biofuels at world-scale volumes. The research permitted by this TERA is critical to the efforts to reach this goal. The TERA subject strain (green microalga) *Parachlorella* sp. STR26155 is engineered with green fluorescent protein (GFP; TurboGFP) for environmental tracking. The aim of this TERA, and the research for which it seeks authorization, in part, is to help SGI establish baseline environmental conditions in and around the test facility, and to evaluate and confirm the sufficiency of control and monitoring equipment and techniques developed for this and other similar outdoor R&D programs. It is also the purpose of this TERA to lay the foundations necessary to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. Gaining insight into how algal strains (top candidates today as well as those to be developed) perform in industrially-relevant settings will inform the design of the technology and ultimately accelerate its development and deployment. It will also reduce the risk of failure that comes with continuing to design a technology without knowing the conditions and constraints it will ultimately face at-scale. This effort will contribute to the development of a globally-relevant Safety, Health & Environment package, or "template", for subsequent TERA and MCAN (TSCA Microbial Commercial Activity Notification) submissions to US EPA and international environmental protection agencies.

There are no other foreseen uses for the subject strain STR26155 except for environmental monitoring. The introduced genetic material, the TurboGFP merely encodes a fluorescent protein that enables tracking of this algal strain. *Parachlorella* sp. STR26155 was not constructed to enable the production of any biofuel compounds or other bioproducts.

VII. GENETIC MODIFICATIONS

1. TurboGFP

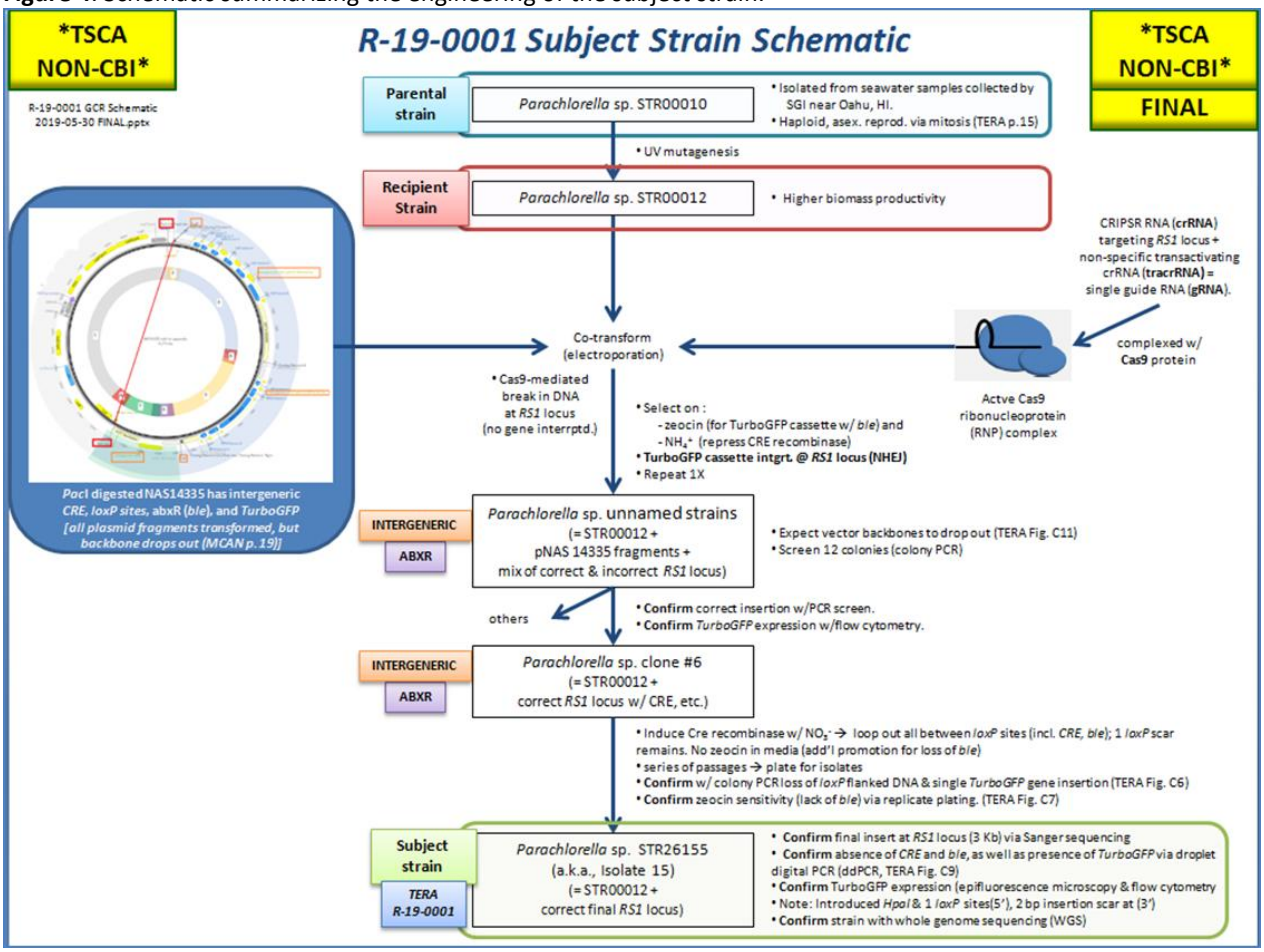
The subject microorganism has an improved variant of the green fluorescent protein from copepod *Pontellina plumata* (CopGFP a.k.a., ppluGFP2; GenBank #AY268072). The vector encoding this improved variant termed TurboGFP, was obtained from Evrogen. An overview of the genetic modification steps was provided by the Genetic Construction Report and presented below (Cameron, 2019).

A summary of the one non-enzymatic protein (TurboGFP) encoded by intergeneric DNA is provided in Table 3 and a more complete description is provide below. A schematic of the genetic construction of the subject strain is provided in Figure 4.

Table 3. Microbiological, genetic, and biochemical details provided for the intergeneric genes used in constructing the subject strain STR26155.

Gene	Source Organism(s)	Function	Promoter	Terminator	Artificially. Synthesized?	Codon-Optimized?
TurboGFP (encoding the green fluorescent protein Turbo GFP; codon-optimized version (for humans) of CopGFP (a.k.a., *ppluGFP2*; GenBank #AY268072). 1 copy integrated at *RS1* locus.	*Pontellina plumata*	Non-enzymatic protein that fluoresces when excited by light (482 nm). Useful for labeling cells and monitoring them.	*ACP1* from *Parachlorella*	*ACP1* from *Parachlorella*	Yes	Yes (*for humans by Evrogen)

Figure 4. Schematic summarizing the engineering of the subject strain.



In brief, the recipient strain was co-transformed with the *PacI*-digested NAS14335 plasmid (both the vector backbone and the fragment containing the *TurboGFP* cassette) and an active Cas9-ribonucleoprotein (RNP) complex that contained a single guide RNA targeting cleavage at the genomic *RS1* locus. Per the TERA, the *RS1* site was selected with the aid of both genome and transcriptome data.

The site was chosen because it was a larger intergeneric (i.e., "between genes" in this context and not indicating presence of genomic DNA from a different genus) region with no detectable transcription. The submitter's goal was to minimize the chance of unintentionally disturbing the function or regulation of nearby endogenous genes due to integration.

The vector backbone (from the commercially available pCC1BAC from Epicentre) which contained a gene that encodes resistance to the antibiotic chloramphenical (*CmR*), several other genes (*HIS3* marker, *sopABC/parABC)*, and an automatic replication sequence-yeast centromere element (ARS-CEN), among other aspects, was expected to drop out as it cannot replicate in *Parachlorella*. Its absence in the subject strain was confirmed by sequencing.

The plasmid fragment containing the *TurboGFP* cassette carried the intergeneric sequence for the gene *ble* that encodes resistance to the bleomycin family antibiotics (e.g., zeocin), the *CRE* gene and associated *loxP* sites (for the Cre-Lox system), as well as the *TurboGFP* cassette. This system targeted insertion of the *PacI* fragment with *TurboGFP* in the recipient strain's *RS1* site.

After co-transformation of the *PacI*-digested plasmid and the active Cas9 nuclease ribonucleoprotein complex, transformants were selected on media with zeocin to ensure integration of the correct *PacI* fragment and ammonium (NH_4^+) to repress CRE recombinase. This was repeated once. The integration of this fragment into the recipient's genome was facilitated by endogenous non-homologous end-joining (NHEJ). Transformants were screened via colony PCR for the correct integration at the RS1 locus. They were also screened for GFP expression with flow cytometry. A clone with the correct profile was selected (#6).

Next, the submitters induced Cre recombinase with nitrate to excise all DNA integrated at the *RS1* site that was between the loxP sites. This included deletion of one of the two loxP sites (one remains in the subject strain). After a series of passages, a culture was plated for isolated colonies, which were analyzed for correct DNA integration and *TurboGFP* expression. Isolate 15 was confirmed to have the correct profile and was designated the subject strain, *Parachlorella* sp. STR26155. At the *RS1* site, the following DNA is integrated (5'→3'): one intrageneric *HpaI* site, one intergeneric loxP site, the *TurboGFP* cassette, and a scar (2 bp insertion).

2. Antibiotic Resistance Markers

Although antibiotic markers, encoding resistance to chloramphenicol (*CmR*) and zeocin (*ble*), were elements of the plasmids used during the strain engineering process, none were present in the final subject strain STR26155. This was by design and was confirmed by colony PCR analysis, digital droplet (dd)PCR, growth (or absence thereof) on zeocin media as appropriate, and whole genome sequencing.

VIII. CONSTRUCT HAZARD ANALYSIS

The potential hazards posed by the genetic modifications and the potential for horizontal and vertical gene transfer of the introduced genetic material were analyzed by McClung (2019).

A. Introduced Genes

1. TurboGFP

The subject strain is engineered to express the TurboGFP for monitoring in the environment. As

previously stated, TurboGFP is a variant of the ppluGFP2 originally isolated from the copepod *Pontellina plumata* (Shagin et al., 2004). TurboGFP, developed by Evdokimov et al. (2006), has a faster maturing brighter fluorescence that the ppluGFP2 from which it is derived. This specific TurboGFP purchased from Evrogen is a version of the TurboGFP developed by Evdokimov et al. (2006) that has been codon-optimized by Evrogen for expression in mammalian cells following the method of Haas et al. (1996). However, this TurboGFP can be successfully expressed in many other systems.

The green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria,* was the first of a number of fluorescent proteins isolated from ocean dwelling-organisms (Shimomura et al., 1962; Johnson et al., 1962). GFP has since been used as an *in vivo* fluorescent marker in a wide variety of microbial, plant, and animal studies (Shagin et al., 2004; Taghizadeh and Sherley, 2008). This GFP does not require substrates or cofactors (Taghizadeh and Sherley, 2008). Chalfie et al. (1994) was the first to report the introduction of the GFP in other organisms, both in the bacterium *Escherichia coli* and in the nematode, *Caenorhabditis elegans*. Yang et al. (1996) elucidated the structure of the green fluorescent protein that consists of 238 amino acids in a cylinder shape comprised of 11 strands of β-sheets with an α-helix inside and short helical segments on the ends of the cylinder. They stated that this structure was a new protein fold not previously observed for any other proteins in nature, which they named the β-can. The cyclization of serine-dehydroxytyrosine-glycine within the α-helix coil in the center of the protein is responsible for the fluorescent chromophore (Cody et al., 1993). It is the barrel structure of the protein that protects the chromophore and provides for its extreme stability. In an interview, several researchers speculated that the barrel structure was also responsible for its lack of toxicity in the cells into which it is introduced (Manning, 1997). The extreme stability of the GFP protein has been demonstrated in numerous studies. As summarized by Haseloff (1998), the fluorescence of this protein has been shown to be unaffected by extended treatment with 6 M guanidine HCl, 8 M urea, or 1% sodium dodecyl sulfate. It was unaffected by 2-day treatments with up to 1 mg/ml of the proteases trypsin, chymotrypsin, papain, subtilisin, thermolysin, and pancreatin. The protein has been shown to be stable up to 65 °C in neutral buffer, and in a pH range of 5.5 to 12. Andersen et al. (1998) reported that the wild-type GFP in *E. coli* had a half-life of greater than 24 hrs. In studies with GFP-tagged *Pseudomonas fluorescens*, Lowder et al. (2000) reported that fluorescence was stable during the entire 6-month incubation period when cells were in the viable but non-culturable (VBNC) state. In the same article, the authors reported that when log-phase cells were killed by UV light, the fluorescence of the supernatant surpassed that of the dead cells which indicated that rather than being degraded, the fluorescent protein was released intact from the cells into the surrounding environment.

The extreme stability of wild-type GFP has proven to be disadvantageous in some applications since it can interfere with the ability to monitor metabolic activity or even cell death. In addition, background interference in environmental samples, particularly in soils, occurs frequently. Consequently, numerous variants of the original GFP with different half-lives or with different emission wavelengths have been developed. An enhanced GFP (eGFP) was developed to alleviate the problems encountered with persistence of the wild-type GFP. Numerous derivatives of GFP and other fluorescent genes with different stability and absorbance/excitation peaks have been created for various applications (Mankin and Thomas, 2001; Zhang et al., 2002). In addition, other fluorescent proteins derived from a group of reef corals belonging to the class Anthozoa such as DsRed2, DsRed Express, DsRed-monomer, AsRed2, HcRed1, AmCyan, ZsGreen, ZsYellow, and p-Timer, and AcGFP1 are now commercially available. Various other fluorescent proteins have been isolated from other ocean-dwelling organisms. As previously mentioned, TurboGFP is a variant of the CopGFP originally isolated from the copepod *Pontellina plumata*. The excitation/emission max of this TurboGFP is 482/502 nm (Cameron, 2019).

There are also reports in the literature suggesting that GFP and some derivatives may present problems in certain constructs in animals and plants (Liu et al., 1999). Aggregation toxicity with GFP has also been reported with a C-terminal addition of a short peptide in *Caenorhabditis elegans* (Link et al., 2006). In

studies using viruses marked with eGFP in retinal cells of rabbits, there was one instance where abnormal morphology was encountered, and the authors speculated that it may have been an immune response rather than toxicity of the eGFP itself (Rex et al., 2005). In most of these viral studies, eGFP up to a several hundred micromolar concentration was not detrimental to retinal cells. In plant cells, Haseloff et al. (1997) reported that GFP could be toxic under high light conditions with high protein expression. In addition, Taghizadeh and Sherley (2008) found that GFP was toxic in rat hepatic adult stem cells, however, yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) worked well as stable marker genes in these cells.

There is one study in the literature assessing the toxicity of GFP fed to rats as pure protein and in a diet consisting of transgenic canola expressing GFP (Richards et al., 2003). The authors reported that oral administration of 1.0 mg of purified GFP/day for 26 days was not toxic to male rats. However, there was a slight, but significant, decline in weight gain in rats fed the GFP canola diet although this was the only parameter affected. The authors reported that GFP was readily degraded in a simulated gastric digestibility study, however, GFP fluorescence was observed in feces of rats fed purified GFP. The authors recommended that their preliminary conclusion that "GFP represents a minimal risk in the food supply" be further investigated in long-term feeding studies. A database search for similarity to known food allergens found only a four amino acid sequence match which was not considered significant which suggested the absence of allergenic epitopes (Richards et al., 2003).

The biological function of these fluorescent proteins in the organisms from which they were isolated is unknown. Long ago, it was hypothesized that fluorescent proteins may serve as protection from strong solar radiation for organisms in shallow waters (Kawaguti, 1944 as cited by Matz et al., 1999). A more recent article (Salih et al., 2000) reported that fluorescent pigments serve a photoprotective function in corals. For organisms in deep waters where light is mostly blue because it is depleted of low-energy components, it has been suggested that the function of the fluorescent proteins may be to provide longer wavelengths than the blue light which may be better for photosynthesis by algal endosymbionts (Schlichter et al., 1994). Shagin et al. (2004) stated that thus far, there seems to more evidence to support photoprotection of endosymbiotic algae, however, the function of these fluorescent proteins is still controversial.

Although there have been studies reporting problems with using GFP in some cells, the TurboGFP used in this *Parachlorella* sp. apparently does not pose problems. According to Evrogen's website (http://evrogen.com/products/TurboGFP/TurboGFP_Detailed_description.shtml), no cytotoxic effects or visible protein aggregation are observed with TurboGFP. TurboGFP was developed by Evdokimov et al. (2006) to overcome some of the problems associated with Copepoda GFPs. These problems include (1) Copepoda GFPs are prone to form aggregates and (2) overexpression of Copepoda GFPs in mammalian cells can lead to the formation of microcrystals that can rupture cell membranes. Therefore, Evdokimov et al. (2006) developed TurboGFP which is a highly soluble, rapidly maturing variant derived from ppluGFP2 (Shagin et al., 2004) (GenBank accession number AY268072). The authors reported that its crystal structure is a β-barrel fold that exists as a dimer in solution at concentrations at least up to 5 mg/ml, and forms tetramers in the crystal form. According to the authors, the TurboGFP chromophore is similar to that of other GFPs that have an abundance of buried charged side chains typical of internalized catalytic centres. It is the residues that contribute functional groups that vary with the different GFPS in the GFP family, but the chromophore-relative positions of important functional groups are conserved. Evdokimov et al. (2006) have succeeded in expressing high levels of their TurboGFP in bacteria, yeast, and mammalian cells.

Evdokimov et al. (2006) also explained the rapid development of the fluorescence. A unique feature of TurboGFP is a water-filled pore leading from the outside of the protein barrel to Y5B of the chromophore. The authors suggested that this pore facilitates oxygen conveyance to the premature

chromophore that speeds up the maturation of the fluorescence.

The TurboGFP apparently functions well as a stable marker gene in this *Parachlorella* algal strain STR26155.

2. loxP site

A single loxP site remains in the genome of the subject microorganism STR26155. The loxP site is from bacteriophage P1 (as is the Cre recombinase enzyme). It is a 34 bp DNA sequence as follows (where N means that the base may change):

13bp 8bp 13bp

ATAACTTCGTATA - NNNTANNN -TATACGAAGTTAT

The two end 13 bp sequences are palindromic. The 8 bp internal sequence is not. In genetic engineering, a pair of loxP sites is used. If the two loxP sites are in the same orientation, the floxed sequence (sequence flanked by two loxP sites) is excised. This well-known system for excision of DNA sequences in between the two loxP sites was used to remove the zeocin resistance gene in creation of the subject strain STR26155. The Cre recombinase and the other loxP site were also lost.

This one loxP sequence remaining in STR26155 does not pose any hazards.

3. Intrageneric *HpaI* site

In addition to the two intergeneric sequences, the subject strain STR26155 also contains an intrageneric *HpaI* site. This is merely a site with a sequence of 5' …GTT | AAC…3' or 3' …CAA | TTG… 5' where restriction enzyme *HpaI* will cut. The presence of this site in STR26155 does not pose any hazards.

B. Potential for Horizontal Gene Transfer

With environmental introduction of genetically engineered microorganisms, the potential for horizontal gene transfer of introduced genes into other microorganism in the environment warrants consideration. Horizontal gene transfer among bacteria is widespread and is responsible for acquisition of a myriad of traits in bacteria such as antibiotic resistance, xenobiotic degradation pathways, and even pathogenesis. Not nearly as much is known regarding horizontal gene transfer in eukaryotes. It has been thought that the barriers to horizontal gene transfer in bacteria are even worse in eukaryotic organisms because of the complexities in their transcription and translation mechanisms (Raymond and Blankenship, 2003). However, from evolutionary analyses, horizontal gene transfer in eukaryotes is known to have occurred. For example, in evolutionary times, it was a primary endosymbiotic event of a cyanobacterium being engulfed that gave rise to the photosynthetic plastid in the common ancestor of the Plantae, such as red and green algae and higher plants (Chan et al., 2012). Likewise, the mitochondria arose from the endosymbiosis and subsequent genetic integration of an alpha-proteobacterium (Keeling and Palmer, 2008). In addition, investigations of the *Chlorella* genome, specifically *Chlorella variabilis*, suggest the ability for *Chlorella* to produce chitinous cell walls as a result of genetic material uptake from algal viruses, prokaryotes, and fungi (Blanc et al., 2010). Eckardt et al. (2010) hypothesized that the *Chlorella* chitin metabolism genes could have been acquired via horizontal gene transfer from viruses. There are other episodes of lateral gene transfer in eukaryotes, such as the phagocytosis by the sea slug *Elysia chlorotica* of the alga *Vaucheria litorea.* The photosynthetic sea slug maintains the algal plastids which continue to photosynthesize for months within the slug (Rumpho et al., 2008).

Very little is known about horizontal gene transfer from one algal species to another. A search of the

literature on horizontal gene transfer in *Parachlorella* did not reveal any studies specifically on horizontal gene transfer in *Parachlorella.* However, there is also evolutionary evidence for horizontal gene transfer in algae. Archibald et al. (2003) found that of the 78 plastid-targeted proteins in the chlorarachniophyte alga *Bigelowiella natans*, approximately 21% of them had probably been acquired from other organisms including streptophyte algae, red algae (or algae with red algal endosymbionts), and bacteria. However, in the green alga *Chlamydomonas reinhardtii,* the homologous genes did not show any evidence of lateral gene transfer. It was suggested that this may be because this green alga is solely autotrophic whereas the *Bigelowiella* is both photosynthetic and phagotrophic. Another instance of potential lateral gene transfer having occurred in algae is the work presented by Raymond and Kim (2012). They found the presence of ice-binding proteins in sea ice diatoms that apparently were essential for their survival in the ice. These protein genes were completely incongruent with algal phylogeny, and the best matches were all bacterial genes. Like bacterial genes, they did not contain introns. There is one example of horizontal gene transfer from an alga to its DNA virus. By phylogenetic analysis, Monier et al. (2009) demonstrated that the transfer of an entire metabolic pathway, consisting of seven genes involved in the sphingolipid biosynthesis, from the eukaryotic alga *Emiliania huxleyi* and its large DNA virus known as EhV had occurred. Hunsperger et al. (2015) reported the conserved presence of the light-dependent protochlorophyllide oxidoreductases (POR) in four different algal taxa (dinoflagellates, chlorarachniophytes, stramenopiles, and haptophytes). The study concluded that the duplicates of stramenopiles and haptophytes *por* genes are a result of horizontal gene transfer from a Prasinophyte alga. A recent study revealed a shared ancestry between the Pedinomonadales and Chlorellales algae after sequencing the chloroplast genome of *Pedinomonas minor* (Pedinomonadales), two trebouxiophyceans, *Parachlorella kessleri* (Chlorallaceae) and *Oocystis solitaria* (Oocystaceae), and comparing the sequences to the chloroplast genome of *Chlorella vulgaris* (Turmel et al., 2009).

There is no information in the literature on horizontal gene transfer specifically with *Parachlorella* nor on the closely related genus *Chlorella*. The intergeneric TurboGFP gene is stably integrated into the chromosome which lessens the likelihood of horizontal gene transfer. It is unlikely that the TurboGFP gene would be transferred to and expressed in other green algae as it does not provide for any selective advantage in the environment. Even if horizontal gene transfer was to occur, the TurboGFP poses low hazards. Although from an evolutionary perspective there is evidence that horizontal gene transfer has occurred in green algae, there are no studies that demonstrate horizontal gene transfer with *Parachlorella,* or the closely related genus *Chlorella,* and other algae.

IX. POTENTIAL HUMAN HEALTH HAZARDS OF THE RECIPIENT MICROORGANISM

The potential human health hazards of the recipient *Parachlorella sp.* STR00012 strain to the general population and to potentially exposed and susceptible subpopulations have been evaluated (Salazar, 2019).

1. General Population

A. Pathogenicity

There is no evidence in the literature that the *Parachlorella* spp. causes infections in humans. However, in extremely rare cases, *Chlorella* has caused infections in humans and other animals. As mentioned previously, since the genus *Parachlorella* was split out from *Chlorella*, it is likely that older reports and studies on *Chlorella* may also apply for the genus *Parachlorella*.

Chlorellosis is the name of this infection by *Chlorella*. It has occurred in limited numbers in sheep and cattle, rarely in humans, and in single cases in a dog, gazelle, beaver, camel, and fish (as summarized by Hart et al., 2014). Animals are infected by exposure of open wounds to contaminated water. In mammals, this disease ranges from localized cutaneous infection, lymph node infection, or dissemination to multiple organs. However, in humans, the three reported cases were cutaneous infections (Jones et al., 1983; Yu et al., 2009; Hart et al., 2014). Chlorellosis in humans is extremely rare. Although *Chlorella* is prevalent globally in fresh water lakes and rivers, in marine waters, and in soil, there have been just three reported cases. Another green alga, *Prototheca* that has been shown to infect humans at a higher rate (more than 100 cases have been reported). However, infection by *Prototheca* is also rare as this alga is widespread in the environment and thus, humans are highly exposed.

The first case of chlorellosis in humans was described by Jones et al. (1983) where a 30-year-old woman developed a persistent infection of a healing operative wound on her right foot after possible contamination by river water while canoeing. The wound was debrided two months later and the infection then treated with antibiotics and wound irrigation. The infection was persistent and healed completely after 10 months.

The second case of *Chlorella* infection was an external infection found in the gangrene tissue from the right foot of a diabetic 59-year-old female (Yu et al., 2009). The *Chlorella* isolate was thought to be *C. saccharophila,* a *Chlorella* strain that uses glucose as a sole carbon source, grows at pH 2-3, and grows at temperatures up to 30°C. The authors stated the strain "could not grow at 37°C in light or darkness. The results suggest that this strain may not normally invade tissues but becomes established and grows on previously infected tissues of external body extremities where the temperature is somewhat lower than normal body temperature."

The most recent case of chlorellosis was reported in Australia in a 30-year-old man in a knee wound contaminated with fresh water dam water (Hart et al., 2014). He developed a *Chlorella* and *Aeromonas hydrophila* infection within two days of exposure and the infection was aggressive and required debridement, negative pressure wound dressings, and antibiotics. However, the wound had healed by the third week with no further complications.

Overall, chlorellosis in humans is extremely rare as there have been just the three reported cases mentioned above, even when the alga *Chlorella* is known to be widespread. The fact that such few *Chlorella* infections have been reported and considering that *Chlorella* is a prevalent alga in fresh water, marine waters, and in soils where humans are frequently exposed to the alga, implies that chlorellosis is quite rare.

B. Toxicity

According to the submission, there are no reports in the literature that any *Parachlorella* or *Chlorella* species, synthesizes or secrets phycotoxins.

The lack of toxin production by *Chlorella* allows it to be used as a popular human nutritional supplement. In addition, *Chlorella* extracts are used in skin care products. *Chlorella* has been proposed as a protein supplement for human consumption (Becker, 2007). *Chlorella* sp. are generally regarded as safe (GRAS) for human consumption. *Chlorella* sp. and *C. protothecoides* flours have GRAS status (GRN 000330; GRN 000519) with the Food and Drug Administration (FDA). In humans, *Chlorella* sp. supplements have shown beneficial effects including improved immune responses, improved healing of the small intestine epithelium, antioxidant action and even anti-tumoral effects (Ramirez-Romero et al., 2010). *C. vulgaris* has been promoted as a prevention of anti-inflammatory responses (Hasegawa et al.,

1999). Morin et al. (1980) have shown inhibitory effects of the unicellular alga *Chlorella* against murine sarcomas. Since *Parachlorella* had previously been classified as *Chlorella*, Buxser (2019) concluded that *Parachlorella* have been used as human food for many years already. Examples of this are shown in Champenois et al. (2015), where *Parachlorella kessleri* might have been used as a food product under the old name, *Chlorella kessleri*.

There is one study in the literature that reported cytotoxicity of algal dietary supplements consisting of a mixture of *Chlorella* sp. and the collective cell biomass from two cyanobacteria, *Arthrospira platensis* and *A. maxima* commonly referred to as *Spirulina* (Heussner et al., 2012). They found extracts from 13 commercially available products sold in Germany were cytotoxic in the A549 cell line with the *Spirulina* being more potent than *Chlorella*. This toxicity, however, was due to contamination of the cyanobacterial and algal cultures by microcystin, a potent toxin produced by the cyanobacterium *Microcystis.* The toxicity was not due to the *Chlorella* or *Spirulina.*

C. Allergenicity

A search of "Parachlorella" AND "sensitization" OR "allergenicity" in PubMed did not result in any published references. As with the previous sections on pathogenicity and toxicity, studies and reports that have been done in the past on *Chlorella*, are very likely to be applicable for *Parachlorella*.

Allergy is the result of a marked increase in reactivity and responsiveness of an immune response to a protein or a low molecular weight compound combined with a larger "self" molecule. However, recent research suggests that not every protein is allergenic (Radauer et al., 2008).

Humans may be routinely exposed to high numbers of algal cells on a daily basis through respiration in both indoor and outdoor environments. Algae and cyanobacteria usually constitute a minority of airborne bioaerosols compared to fungi, pollen, and bacteria; however, in certain cases the quantity of airborne algal particles can far exceed that of fungi spores and pollen grains (McGovern et al., 1965). Brown et al. (1964) found over 3000 algae/m^3 in samples taken from a car moving through a dust cloud in Texas. Schlichting (1969) found < 8 algal cells/ft^3 in air sampled in Texas, Michigan, and off the North Carolina coast and calculated that breathing 240 algae cells per hour was possible for a maximum daily uptake of 2880 algal and cyanobacterial cells. In a summary of the existing literature on airborne algae during the years 1910 - 1968, a total of 187 taxa of algae and protozoa were found. Several species of *Chlorella* were sampled directly from the air including *C. ellipsoidea, C. pyrenoidosa, C. vulgaris,* and *Chlorella* sp. (Schlichting, 1969). Bernstein and Safferman (1970) also found 18 different genera of algae in house dust collected from 41 homes of which *Chlorella* was the most frequently encountered algae, followed by *Chlorococcum, Schizothrix, Planktosphaeria, Chlamydomonas, and Anabaena.*

There is evidence from human studies that *Chlorella* can induce hypersensitivity responses in some individuals. Tiberg et al. (1995) tested Swedish children for allergy to *Chlorella* using three methods: the radioallergosorbent test (RAST), skin prick tests (SPTs), and conjunctival provocation tests (CPT). These tests detect specific IgE antibodies to determine whether a subject is sensitized to the substance. No *Chlorella*-specific IgE antibodies were found in the sera from the 94 children from the general population (group 1 – no allergy symptoms). In a group of children that had been referred to an outpatient pediatric allergy clinic (group 2), nine of the 129 children had positive wheal reactions with the *Chlorella* extract in SPTs. Sera from seven of these children with positive SPTs results were available for analysis of IgE antibodies. Two of the seven were positive for IgE-specific antibodies to Chlorella. Seven of 23 mold-sensitive children (group 3) had positive SPTs to *Chlorella.* Six patients with SPT positive results and two of the 16 patients with negative SPT results had positive RAST results. All patients with positive SPT results showed some reaction in CPTs with *Chlorella* extract (5 mg dry weight/ml). These data demonstrate that only children that are sensitized to many common allergens also were sensitized to

Chlorella and no specific symptoms related to *Chlorella* sensitization were observed. These data suggest that *Chlorella* is a weak allergen.

Similarly, Bernstein and Safferman (1966) tested two species of *Chlorella, C. vulgaris* and *C. pyrenoidosa,* two species of *Chlorococcum, C. botryoides* and *C. macrostigmatum*, *Scenedesmus basilensis,* and *Ankistrodesmus falcatus* var. *acicularis* for their potential to elicit cutaneous reactions in atopic patients, i.e., those with a genetic predisposition for developing allergic hypersensitivity reactions. They found that of 79 atopic patients tested with algal extracts, 47 also gave positive skin reactions while non-atopic individuals did not show positive skin reactions. Additional tests with *C. vulgaris* for bronchial mucosa tests resulted in clinical wheezing. Interpretation of this study is greatly limited by lack of antigen quantification or understanding of the cellular and molecular mechanisms involved and small sample sizes.

The database Allergome lists *Chlorella* as an allergen, however the WHO/IUIS Allergen Nomenclature database (Allergen Nomenclature (IUIS); http://www.allergen.org) does not. Based on a review of outdoor allergens, Burge and Rogers (2000) stated that algae do not seem to be a source of major outdoor allergens. The lack of any more recent studies in the literature regarding potential allergenicity of *Chlorella* over the past several decades suggests that it is not an important environmental allergen.

There is a single report of occupational asthma in a pharmacist induced by exposure to a fine dust powder of *Chlorella* while making chlorella tablets for human consumption (Ng et al., 1994). It was suggested that the causative agent in this chlorella-induced asthma was pheophorbide-a, which is a breakdown product of chlorophyll, and its ester, or some other protein component. Pheophorbide-a and its ester are formed by the reaction of the chlorophyllase enzyme during the drying process of the moist *Chlorella* cells with heated air at 90°C. Given that the hypersensitivity response was induced by fine, dry dust created by high heat, the relevance of this report of occupational asthma to exposures of live moist *Chlorella* cells in bioaerosols during this field test is questionable.

D. Other Effects

Chlorella has also been reported to cause photosensitization, which is development of abnormally heightened reactivity of skin or eyes to sunlight, in those who took *Chlorella* as a dietary supplement (Jitsukawa et al., 1984). In addition, protein components of *Chlorella* such as a breakdown product of chlorophyll, pheophorbide-a and its ester that are recognized as photosensitizers may contribute to adverse reaction in the kidney (Yim et al., 2007). However, this photosensitization resulted from ingestion of algae which is not relevant to exposures in this TERA field test with the closely-related *Parachlorella*.

2. Potentially Exposed and Susceptible Subpopulations

Potentially exposed individuals are workers at the SGI facility. Susceptible subpopulations that warrant consideration differ whether in relation to potential pathogenicity or allergenicity of *Parachlorella.* In terms of pathogenicity, susceptible subpopulations would include those whose immune systems are not fully competent such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapies. Susceptible populations for allergenicity concerns are atopic individuals which are those with a genetic predisposition toward developing hypersensitivity reactions to environmental antigens.

A. Pathogenicity

Parachlorella has not been reported as causing any infections in humans. However, there are three reports of *Chlorella* sp. infections in humans originating in open wounds after exposure to contaminated water. Chlorellosis is extremely rare even though humans are routinely exposed to *Chlorella* as it ubiquitous in the environment in fresh waters, marine waters, and in soils, and even found in the indoor environment in house dust. Thus, there is little concern even for those with not fully competent immune systems as they too are routinely exposed to *Parachlorella* sp. Dermal contact of workers to the alga in the open miniponds is not expected as workers will be wearing personal protective equipment required by SGI regulations (e.g., gloves, safety glasses, long pants, and steel-toed shoes) when handling the algae.

B. Toxicity

In regards to toxicity, there is low concern for potentially exposed or susceptible subpopulations as well as the general population as *Parachlorella* is not known to produce any phycotoxins.

C. Allergenicity

There may be some concern for allergenicity with potentially exposed and susceptible subpopulations if any workers are atopic individuals that are prone to developing hypersensitivity reactions even though *Chlorella* has been characterized as being a "weak" allergen (Tiberg, 1995). Bioaerosols containing algal cells are expected to be generated during the growth of the algae in open raceway ponds so some inhalation of the submission strain *Parachlorella* sp. STR00012 is expected. The general human population does not appear to suffer allergenicity symptoms from exposure to *Chlorella* since *Chlorella* is ubiquitous in the environment in fresh water, marine waters, and soils, and even occurs in house dust so humans routinely inhale *Chlorella* cells. Based on a review of outdoor allergens, algae do not seem to be a source of major outdoor allergens (Burge and Rogers, 2000). It is unlikely that atopic individuals would choose to work with algae given their predisposition to developing hypersensitivity reactions. However, if atopic individuals work at the facility, allergenicity symptoms could be alleviated by the use of respirators (APF50 respirators with P100 filters that removes 99.97% of exposure to microorganisms).

X. POTENTIAL HUMAN HEALTH HAZARDS OF THE SUBMISSION MICROORGANISM

The potential human health hazards of the submission microorganism *Parachlorella* sp. STR26155 to the general population and to potentially exposed and susceptible subpopulations have been evaluated (Salazar, 2019).

1. General Population

The concern for pathogenicity or toxicity associated with the introduced gene is low. As described by the submitters and the Construct Hazard Analysis (McClung, 2019), the introduced DNA coding for TurboGFP, is not expected to introduce any other phenotypic change in the recipient microorganism and does not impart or enhance any harmful traits beyond what may be present in the recipient strain.

Although resistance genes to the antibiotics chloramphenicol and zeocin were used in the development of the subject strain STR26155, they are not present in this final submission strain.

2. Potentially Exposed and Susceptible Subpopulations

The genetic modifications of the recipient to make the submission strain *Parachlorella* sp. STR26155 strain do not pose adverse human health effects to potentially exposed and susceptible subpopulations just as they do not to the general human population. The introduced TurboGFP does not pose pathogenicity, toxicity or allergenicity concerns to humans.

XI. POTENTIAL ECOLOGICAL HAZARDS OF THE RECIPIENT MICROORGANISM

The potential ecological hazards of the recipient and the submission microorganisms have been evaluated by Nguyen (2019). According to the *Parachlorella* literature review by Buxser (2019), there are no records of adverse impacts of the genus *Parachlorella* to any terrestrial plants or animals. There are also no records of toxicity or pathogenicity of *Parachlorella* to any aquatic plants or wildlife, although there may be potential for population effects related to competition/biogeochemistry (Buxser, 2019). In a broader context, the interactions of algae in aquatic and terrestrial environments and their role in aquatic food webs were discussed in a previous risk assessment for an algal submission by McClung (2013).

Parachlorella spp. have been isolated from a wide range of freshwater (also saltwater) environments worldwide, including California (proposed TERA test site) (Figure 5; Buxser, 2019). Despite this worldwide prevalence of *Parachlorella* spp., there have been no reports of adverse bloom formation from this genus. Like *Chlorella* spp., *Parachlorella* spp. are very tolerant to various growth conditions including extreme temperatures, pH, salinity, high nutrient and heavy metal concentrations (Huss et al., 1999; Juarez et al., 2011; Shimura et al., 2012; Whitton et al., 2015).

Figure 5. Locations where *Parachlorella* spp. have been isolated (Buxser, 2019)

Three genera of green algae, *Chlorella, Chlamydomonas,* and *Scenedesmus* are the dominant green algae in many aquatic habitats and are frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998). *Chlorella* is a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007).

Chlorella is resistant against a number of environmental stressors related to its metabolic versatility, and thus is able to cope with shortages of nutrients and water. This genus has a high tolerance to temperature and can easily live in both terrestrial and aquatic ecosystems. Members of the genus *Chlorella* are found in freshwater natural and artificial water habitats throughout the world (Trainor, 1998) and some species can even thrive in polar regions and hot deserts (Hodac et al., 2016). *Chlorella* have been reported from nearly all soil types, including: desert soil crusts, where it was one of the most common genera found across 4 of 7 different biomes sampled across the Namibian-Angola border (Budel et al., 2009); humic tropical soils in India, biofilms covering natural and artificial subaerial substrates and dwell in soils, and polar desert soils in Antarctica and Artic (Hodac et al. 2016). They can be also grown in wastewater and used for the removal of metals (De-Bashan et al. 2008). Phylogenetic analysis (using SSU and ITS2 rDNA sequencing) has shown their polar, temperate and tropical distribution, in addition to demonstrating that even polar isolates are closely related to temperate ones (Hodac et al., 2016). Hodac et al. (2016) concluded based on sequence similarities that *Chlorella* might be capable of intercontinental dispersal; however, they acknowledge that their actual distributions may exhibit biogeographical patterns but requires further research. Although most *Chlorella* species are naturally free-living, some are known photosynthetic symbionts, such as one species known to be a symbiont of the unicellular protozoa *Paramecium bursaria* (Blanc et al., 2010).

Microalgae, depending on specific species characteristics and culture conditions, will employ different

metabolic pathways for growth. *Chlorella* (and also *Parachlorella)* may be capable of growth under autotrophic, heterotrophic and mixotrophic conditions (Kim et al., 2013). Under autotrophic conditions microalgae fix CO_2 to organic matter using light energy, which results in the reduction of CO_2. Heterotrophic microalgae can grow using organic carbon a sole carbon source without the need for light. Mixotrophic microalgae can metabolize both organic and inorganic carbon using metabolic characteristics of both auto- and heterotrophs; using energy produced from organic sources for cell synthesis and storage of chemical energy converted from light energy (See Table 4). Requirements for nitrogen and phosphorus seem to also differ between all three growth types. For example, Kim et al. (2013) reported higher requirements under heterotrophic growth conditions than for auto- or mixotrophic growth conditions. Autotrophic microalgae growth has been shown to be lower than that of heterotrophic or mixotrophic types, thus making it possible and advantageous to grow microalgae at high rates in lightless conditions that match or exceed autotrophic growth.

Table 4. Energy and carbon source of microalgae by growth type (adapted from Kim et al., 2013).

Growth type	Energy Source	Carbon Source
Autotroph	Light	Inorganic
Heterotroph	Organic	Organic
Mixotroph	Light and organic	Inorganic and organic

The growth requirements of *Parachlorella* are thought to be similar to those of *Chlorella* which are relatively simple, and do not differ greatly from that of other microalgae (Eyster 1967; Huss et al., 1999). For example, many *Chlorella* spp. and *Parachlorella* spp. can readily grow in Bold's Basal Medium, (containing low concentrations of phosphate, nitrate, sulfate, borate, K, Ca, Mg, Na, Zn, Mn, Mo, Cu, Co, and Fe) at pH 6.8 (Krienitz and Bock 2012). As mentioned earlier, *Parachlorella* can also utilize various energy and carbon sources. *Parachlorella*'s broad distribution can be attributed to these simple growth requirements, along with its tolerance to a variety of environmental conditions, including extremes. Examples can be seen with *Parachlorella kessleri* (previously *Chlorella kessleri*) and a previously unknown *Parachlorella* isolate found downstream from the Fukushima Daiichi Nuclear Plant (Juarez et al., 2011; Shimura et al., 2012). *P. kessleri* was isolated from a mesothermal acidic pond in Argentina with a high sulfuric acid concentration (Juarez et al., 2011). The optimal growth conditions of this isolate were: pH (2.5-3), NaCl (1-2%), temperature (34-36°C). The isolate found near the Fukushima Daiichi Nuclear Plant could grow at high temperatures and withstand a wide range of pH (3-11), along with the ability to grow in fresh or salt water (Shimura et al., 2012). *P. kessleri* was also found at a coal-fired thermoelectric plant in Brazil where growth was measured at several concentrations of CO^2: 6%, 12%, and 18% (de Morais et al., 2007).

In a wastewater adaptation study, Osundeko et al. (2014) tested the growth of *P. kessleri* and five other species from four genera, including two *Chlorella* species, in secondary-treated municipal wastewater during an 8-week period. The results of the study showed that *P. kessleri* was one of the best at acclimating to growth in wastewater, along with its efficiency in the removal of nitrogen and phosphorus (Osundeko et al., 2014).

The occurrence of many species of algae throughout the world suggests that algae can readily disperse over great distances. Studies on microalgae have shown that most species are globally distributed (cosmopolitan) but some species have more restricted distribution due to environmental factors such as temperature or humidity, and limited dispersal mechanisms (Kristiansen, 1996a). In a review of data on the distribution of coccid green algae in the environment, Komárek and Comas (1984) said that the distribution is dependent on the specific environmental requirements of the taxon. They stated that "Chlorococcalean algae (*Parachlorella* and *Chlorella* belong to this group) are traditionally supposed to be organisms of cosmopolitan occurrence. Many species occur, indeed, in various regions all over the world, but, many other taxa occur in geographically limited areas, mainly in either the northern or the

tropical countries".

Chlorella, and likely *Parachlorella,* have a few known predators that are of concern for open pond cultivation, among them rotifers and some bacteria. Various strategies are being investigated for loss prevention of *Chlorella* cultures (e.g., pond crashes). Many are exploring the use of biomolecule production in algae for improving their innate defense against bacteria and rotifers (Sayre et al., 2015). Sayre et al. (2015) has examined the use of various antimicrobial peptides (AMPs) to protect against rotifer and bacterial infection and its effect on algae growth, while others are looking at genetic engineering endogenous compounds that can be produced and released by the various strains to prevent infection of the cultures. Cultivation pond experiments with *Chlorella* have demonstrated that algal-associated bacterial communities shift over time, and crashes of cultures are often associated with *Vampirovibrio chlorellavorus* infection. Therefore, various groups are working to develop PCR-based tools for monitoring contaminants. The National Alliance for Advanced Biofuels and Bioproducts (NAABB), for example, has designed primers that amplify a 1500 nucleotide region of the 18S rRNA gene from three major classes of algae: Bacillariophyceae, Eustigmatophyceae, and Chlorophyceae. "These amplicons can be sequenced for definitive identification of strains, or they can be digested with a restriction enzyme to generate allele-specific fragmentation patterns for rapid, inexpensive characterization of strains and cultures. This work provides molecular tools to detect and monitor algal population dynamics and clarifies the utility, strength, and limitations of these assays. These include tools to identify unknown strains, to routinely monitor dominant constituents in cultures, and to detect contaminants constituting as little as 0.000001% of cells in a culture. One of the technologies examined was shown to be 10,000X more sensitive for detecting weeds than flow cytometry" (Sayre et al., 2015). In addition, NAABB is also looking at developing molecular monitoring tools for tracking bacteria that are associated with the cultivation of different microalgal species as a means of determining the health of the culture and mitigating pond crashes.

Although some genera in the class Trebouxiophyceae can cause harmful algal blooms (HABs), the genus *Tetraspora*, *Parachlorella,* and *Chlorella* are not associated with harmful algal blooms (HABs). The genera *Chlorella* and *Parachlorella* are not listed as a harmful species, including in UNESCO's list of harmful micro algae (webpage: http://www.marinespecies.org/hab/ visited May 2019). These genera thrive in higher temperatures than other common species in moderate nutrient-loaded environments so they are known to bloom later in the year (Elliot et al., 2006; Cordero et al., 2011). Although *Chlorella* has the potential of producing dense blooms, to date there is no available literature showing that *Chlorella* blooms have caused any adverse effects (Ryther, 1954). The only references that cite a *Chlorella* bloom event (Pan et al., 2011; Li and Pan, 2013) are based on erroneous interpretation of a paper by Ryther (1954) who mentions *Chlorella* (but not in association with the observed decimation of the oyster industry on Long Island), which was attributed to eutrophication stimulated by duck farm effluents which led to blooms of *Nannochloris atomus* and *Stichoccocus sp.* So, to date, there has been no recorded HAB event associated with *Chlorella sp.*

However, one area of potential concern is the ability of some *Chlorella sp.* to produce chlorellin, an antibiotic-like substance that can inhibit its own growth and that of $Gram^+$ and $Gram^-$ bacteria. Older literature has demonstrated that *Chlorella* (and thus possibly *Parachlorella*) can produce substances that are inhibitory to the growth of other algae, such as *Nitzschia frustulum* (Rice, 1949). These experiments simply exposed competing algae to the exudates of *Chlorella sp.* and did not characterized the specific molecule(s) associated with the inhibitory effect. Therefore, it is theoretically possible that Para*chlorella* may have a survival advantage if it is able to produce chlorellin or some other molecule inhibitory to other algae and to bacteria. However, there is no literature suggesting that *Parachlorella* produces inhibitory compounds.

1. Potential Effects of Parachlorella sp. on Terrestrial Mammals

Indirect effects on terrestrial mammals can result from ecosystem-level disruptions through the establishment of novel strains of *Chlorella* in freshwater habitats. Disruptions of these freshwater ecosystems through the introduction of new algal strains could result in harmful algal blooms (HAB) (Anderson et al., 2002). HAB events can disrupt highly complex stochastic mixing and flushing patterns and increase the eutrophication potential of waterways (Anderson, 2002; Hoagland et al., 2002). Disruptions of these waterways can negatively affect terrestrial wildlife that rely on freshwater ecosystems for food or habitat. However, as noted above, there is no literature indicating that *Chlorella* (or *Parachlorella*) has ever been responsible for HABs.

There are no reports in the literature on animal infections caused by *Parachlorella*, but effects from exposure to *Chlorella* sp., although rare, have been reported leading to infection of open wounds. Pathogenic infection of tissue by *Chlorella*, known as chlorellosis, has been reported in numerous species of mammals including gazelles, sheep (both adults and lambs), cattle, dromedaries, dogs and beaver (Cordy, 1973; Kaplan et al., 1983; Le Net et al., 1993; Philbey, 2001; Haenichen et al., 2002; Quigley, et al., 2009; Ramirez-Romero et al., 2010). Documented cases of chlorellosis are rare and are typically the opportunistic infections resulting from contamination of wounds or dissemination from the gastrointestinal tract following oral ingestion of stagnant water or sewage-contaminated water (Kaplan et al., 1983; Zakia et al., 1989; Philbey et al., 2001; Haenichen et al., 2002; Ramirez-Romero et al., 2010). Effects of chlorellosis in terrestrial mammals include the formation of lesions in the skin, liver, lungs and lymph systems accompanied by a characteristically green discoloration of the affected organs (Ramirez-Romero et al., 2010). Similar to infections in humans, ingestion of *Chlorella* has been shown to result in skin sensitivity, although organismal-level effects on terrestrial wildlife as a result of this effect are uncertain (Jitsukawa et al., 1984). While the majority of cases of chlorellosis have been reported in immunosuppressed individuals, several cases indicate that chlorellosis can occur in non-immunosuppressed mammals (Kaplan et al., 1983; Philbey et al., 2001). There is limited information available to characterize chlorellosis infections in terrestrial wildlife so there is uncertainty related to the mechanism of infection and which species of *Chlorella* are most likely to exhibit pathogenicity.

2. Potential Effects on Plants

There is no information in the literature that suggests any negative effects of *Parachlorella* (or *Chlorella)* on aquatic or terrestrial plants.

XII. POTENTIAL ECOLOGICAL HAZARDS OF THE SUBMISSION MICROORGANISM

As discussed in the Ecological Hazard Assessment, (Nguyen, 2019) the introduction of TurboGFP is expected and was shown by the submitters to have no discernable phenotypic differences in the subject strain *Parachlorella* sp. STR26155 relative to the recipient strain *Parachlorella* sp. STR00012. Various growth tests were performed to ensure that the subject strain has no greater propensity to impact primary productivity than the recipient strain. GFPs, from various sources, have been utilized as a reporter protein and are well-characterized in many host systems with minimal impact to their phenotype. The TurboGFP is not expected to introduce any new hazard concerns in the subject microorganism *Parachlorella* sp. STR26155 compared to the recipient strain.

XIII. POTENTIAL SURVIVAL OF THE SUBMISSION MICROORGANISM

As mentioned previously, *Chlorella* (and probably *Parachlorella)* is one of the most dominant green

algae in many aquatic habitats and can be frequently isolated from marine, fresh water, soils and air samples, as it can tolerate a wide range of environmental conditions (Trainor, 1998). As shown in Figure 5 above, *Parachlorella* too has been isolated across the globe. *Parachlorella* is also a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007). *Parachlorella* is also likely to be an aeroalgae due its small unicellular nature.

In addition, Tiffany (1951), defined algae into nine different groups based on preferred habitat; including edapophytes (soil algae), aerophytes (aerial algae), endophytes (living within plant tissue) and endozoophytes (living inside animal hosts), all of which are habitats in which different *Chlorella* species have been known to thrive in. Lists of soil algae have been compiled across the country and the world, showing their diverse distribution, and frequently include *Chlorella* (Metting, 1981). Soil bound *Chlorella* species appear to tolerate high levels of radiation than other more complex terrestrial life forms (Metting, 1981). Trainor (1962) was even able to show that *Chlorella* is able to survive desiccation for one hour at 130˚C. Despite their high tolerance to a variety of stressors, Metting (1981) showed that various *Chlorella* strains are negatively affected by a variety of herbicides and insecticides, and thus could be used to minimize the dispersal of *Chlorella* (potentially *Parachlorella)* cultured in outdoor ponds. Since the genus *Parachlorella* was split out from *Chlorella*, it is likely to also survive desiccation and other stressors mentioned above.

However, little research is available that directly shows that *Parachlorella* sp. STR00010/STR26155 can survive as well as many other species in the same genera, and more research is required on the wild type strain to determine the true potential for survival posed by new strain. Ultimately, the survival characteristics are not expected to change from the wild type recipient to the submission strain.

XIV. DESCRIPTION OF THE FIELD TEST SITE

The field testing of *Parachlorella* sp. STR26155 will be carried out at the Synthetic Genomics, Inc. California Advanced Algae Facility (CAAF) in Calipatria, CA. The CAAF is located on private land approximately three miles east of the Salton Sea in the unincorporated area of the County of Imperial, California. The physical address is 250 West Schrimpf Road, Calipatria, CA, 92233. The legal land description is: the northwest and southwest quarters of Section 19, Range 14E, Township 11S.
The facility's approximate geographic coordinates are N 33.198491 W 115.558857. It is bound on the north by McDonald Road and the Imperial Irrigation District's (IID) "O" Lateral and on the south by Schrimpf Road and the IID's "O" Drain. The "O" Lateral is fed by the All American Canal. Regional access is provided from State Route 111, via McDonald Road. An existing driveway entrance is located on Schrimpf Road. A six-foot chain link fence surrounds the property, with a controlled-access gate on Schrimpf. An east-west six-foot chain link fence divides the property into two forty-acre sections. The northern section is not currently active. The site is staffed with 15-20 full and part time employees.
The specific area within the CAAF that will be utilized to operate the two 100,000-liter (0.1 acre) ponds is approximately one-half acre in size, located on the southwest part of the facility. See Figures 6-8 below.

Elevation and slope - The site rests at an elevation of 220 feet below mean sea level, on a plot of land that is exceptionally flat, sloping very gently downward to the west. For reference, the surface of the Salton Sea is approximately 227 feet below mean sea level. A drainage study was commissioned by SGI in 2014.

Proximity to water bodies - The site is located near (~three miles) to the Southeast corner of the Salton Sea (Figure 6). The nearest fresh water source (at a distance of ~1.5 miles) is the Alamo River, located to the Southwest of our facility. The site uses production water provided by the Imperial Irrigation District (IID), which sources their water from the Colorado River. The IID transports river water from Yuma AZ, utilizing various open channel irrigation canals that network throughout the Imperial Valley. The site is designated as a zero-discharge facility meaning that none of the water taken onto the site is released back into in the local water system (with the exception of rainwater not falling into a pond or collection basin).

Prevailing winds - The prevailing winds at the site arise from the southeast. Summarized daily averages for one calendar year shows the strong frequency of winds from this direction. There is a less frequent, but moderately more intense wind pattern with winds coming from due west. This variation in the prevailing wind is most prevalent in spring-time months, although not exclusively so. Hourly averages for the months of May through August (the anticipated months of the experimental release application) show a similar pattern to the yearly plot of daily values. Winds are predominantly from the southeast and generally more moderate during these months of the year.

Figure 6. Aerial view of SGI's CAAF facility and local vicinity. The CAAF facility is bordered by the red rectangle. Environmental sampling stations are labeled and marked with red bulls-eyes.

Figure 7. Topographic map of project area with neighboring municipalities, roads, water and geothermal features.

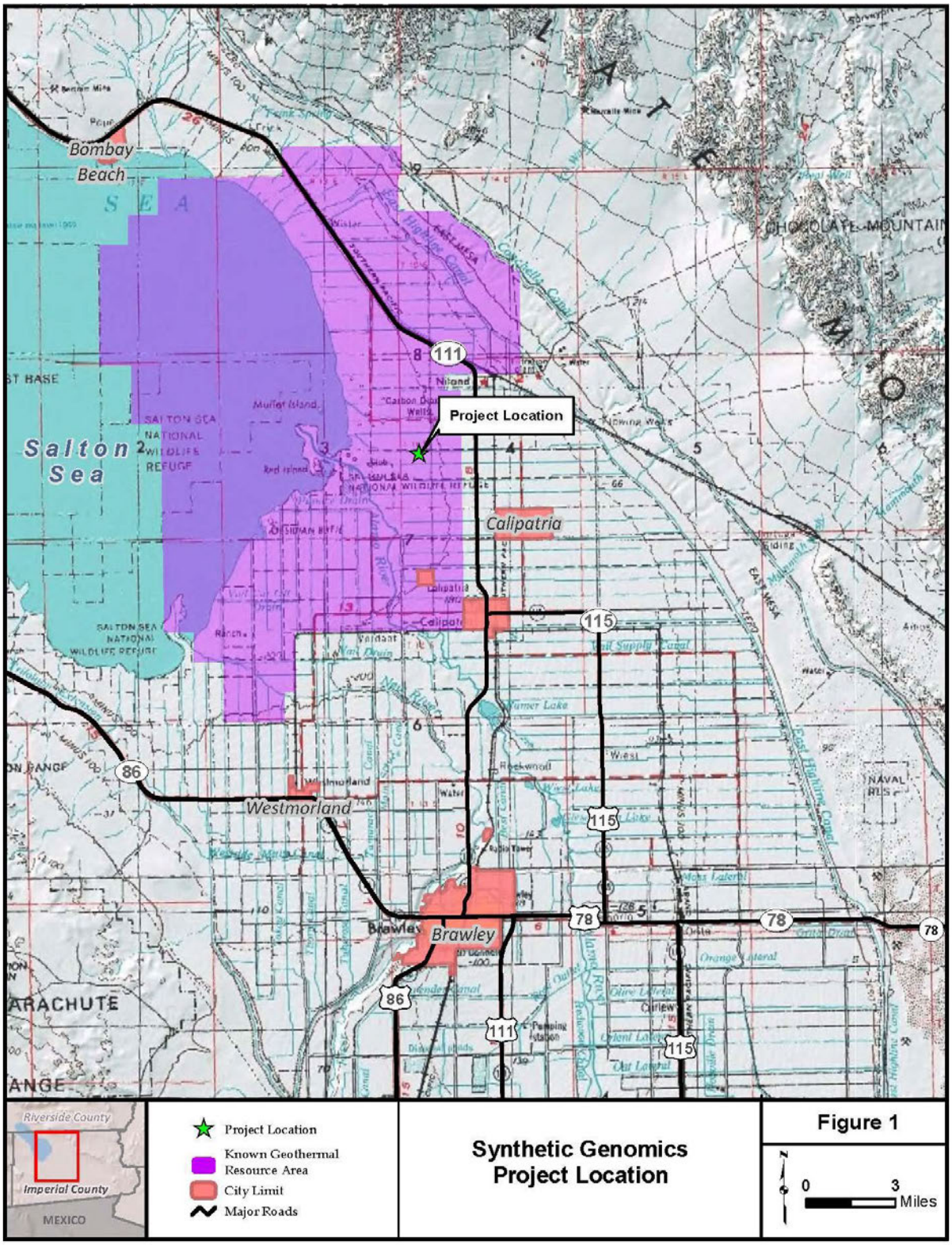
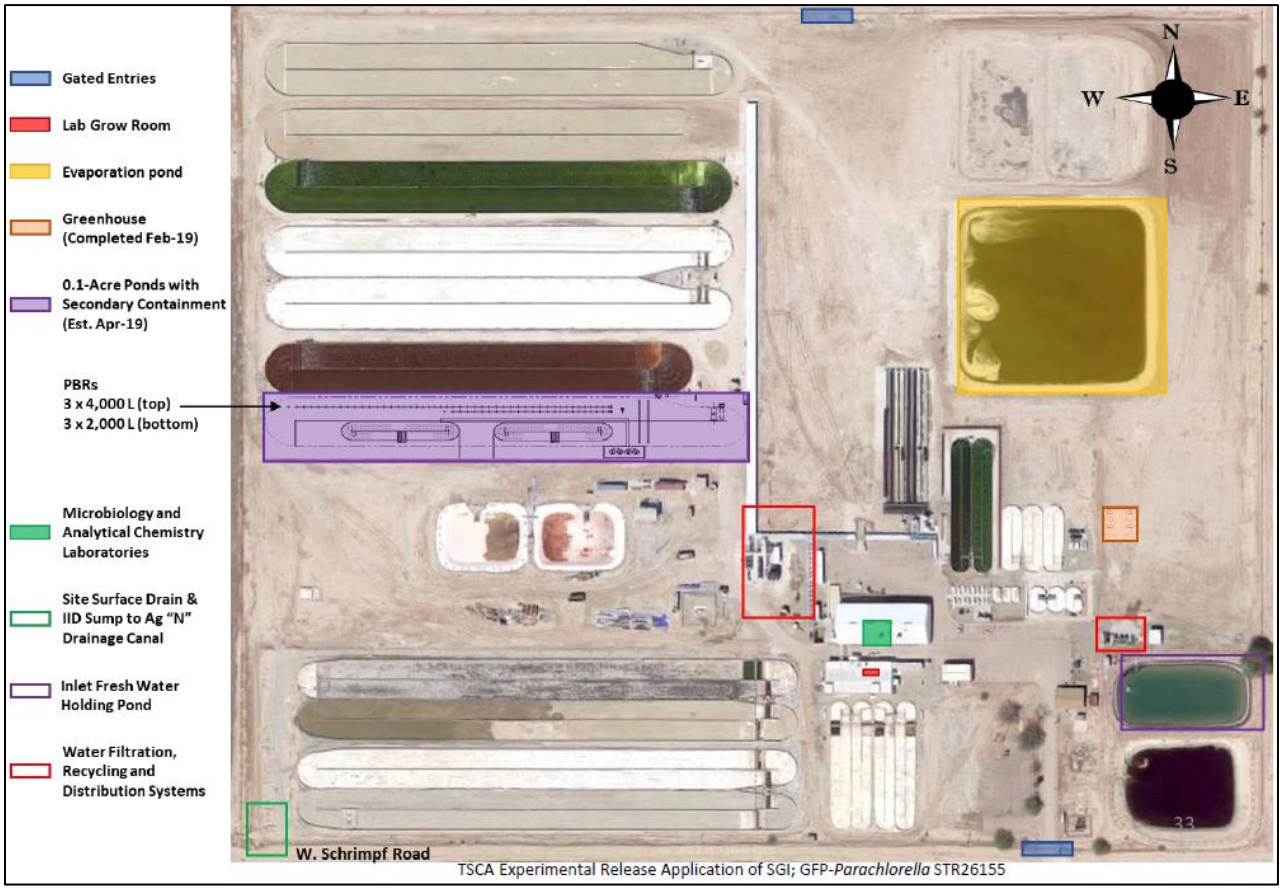


Figure 8. Satellite photo of CAAF facility (Feb-15). Structures and facilities of particular importance to this application are identified. The 0.1-acre ponds subject to this application and the greenhouse were built in 2019.



XV. STUDIES TO BE CONDUCTED TESTS AT THE FIELD TEST SITE

The purpose of this field trial is to work with an engineered alga in open ponds at a scale larger than prior work which begins to approach the expected scale needed for future commercial viability. The submitter proposed to grow the subject strain in open "raceway" ponds" (described in-detail below) of 0.1-acre surface area in a manner that reasonably mimics what future production processes may be. This will enable the collection of real-world data on the potential for their algae to disperse, establish, and impact the local environment. This data is crucial to inform future applications wherein engineered algae with improved productivity phenotypes will be tested at increasing scale.

Seed stocks will be maintained in a dedicated grow room and transferred only between sealed containers during the scaling process. Once at least 100 L of seed has grown to a density of at least 1.0 g/L, the seed stock will be utilized to inoculate the 2,000 L and 4,000 L photobioreactors (PBRs) at a density of approximately 0.1 g/L. Once the PBRs reach a density of at least 1.0 g/L, they will inoculate one of the 0.1-acre ponds at a target operational starting density of 0.1 g/L (Figure 8). These ponds will then run for one week each. At the end of a week of growth, the ponds will be deactivated and disposed.

Details of monitoring endpoints, procedures, and timelines are provided in R-19-0001. Briefly, while running the 0.1-acre raceway ponds in a production-like mode (although still for R&D purposes, and at a significantly smaller scale than full-scale biofuel production ponds) SGI will regularly sample multiple sample types from a variety of sites (e.g. bioaerosols, trap ponds, CAAF production ponds, local environmental sampling) to provide data on the potential release of the engineered alga from the

experimental ponds. SGI will conduct active monitoring for one week prior to the start of open engineered alga cultivation, during the entire course of the experiment, and for 2 weeks following termination of the engineered alga ponds. During this active monitoring period, one type of endpoint will be the five 350 L "algae-trap" ponds established to help assess the dispersion capability of the subject organism. Additionally, SGI will sample regularly from all other ponds on site that are in active use and assay for the presence and abundance of the subject strain. Lastly, regular bio-aerosol samples will be collected and similarly assayed for the presence and abundance of the subject strain. Both during the active monitoring, and for one year following first inoculation, SGI will continue to carry out passive monitoring consisting of monthly sampling from our established environmental stations.

Samples will be collected daily for the CAAF Lab to perform growth measurements as described in Table 5. Briefly, these measurements will include optical density (OD730), ash-free dry weight (AFDW), photosynthetic efficiency (PAM), total organic carbon (TOC), fatty acid methyl ester composition (FAME), microscopic analysis and metagenomic analyses. Excess samples will be disposed of in 0.5% sodium hypochlorite. The culture will be inoculated with media containing nitrogen, phosphorus, and trace minerals.

Table 5. Sampling frequency and measurement type. (R-19-0001)

Measurement Type	Frequency	Sampling Location
OD_{750}	Daily	0.1 Acre ponds, PBRs
AFDW	Daily	0.1 Acre ponds, PBRs
Pulse Amplitude Modulation (PAM; Photosynthetic efficiency)	Daily	0.1 Acre ponds, PBRs
Lipid Content (FAME)	Daily	0.1 Acre ponds, PBRs
Total Organic Carbon (TOC)	Daily	0.1 Acre ponds, PBRs
Microscopy	Bi-weekly	0.1 Acre ponds, PBRs
Microbial Ecology	Bi-weekly	0.1 Acre ponds
pH Measurement	10 minutes	0.1 Acre ponds, PBRs
Water Temperature	10 minutes	0.1 Acre ponds, PBRs
Dissolved Oxygen	10 minutes	0.1 Acre ponds, PBRs
Conductivity	10 minutes	0.1 Acre ponds, PBRs
Air Temperature	10 minutes	Weather Station
Wind Speed	10 minutes	Weather Station
Wind Direction	10 minutes	Weather Station
Photosynthetically Active Radiation	10 minutes	Weather Station
Precipitation	10 minutes	Weather Station
Relative Humidity	10 minutes	Weather Station
Trap Pond Samples	Bi-weekly	378 L traps

XVI. EXPOSURE ASSESSMENT

For a detailed account of potential releases of the production microorganism during laboratory propagation, growth, and waste disposal, see the Engineering Report (Hollinshead, 2019).

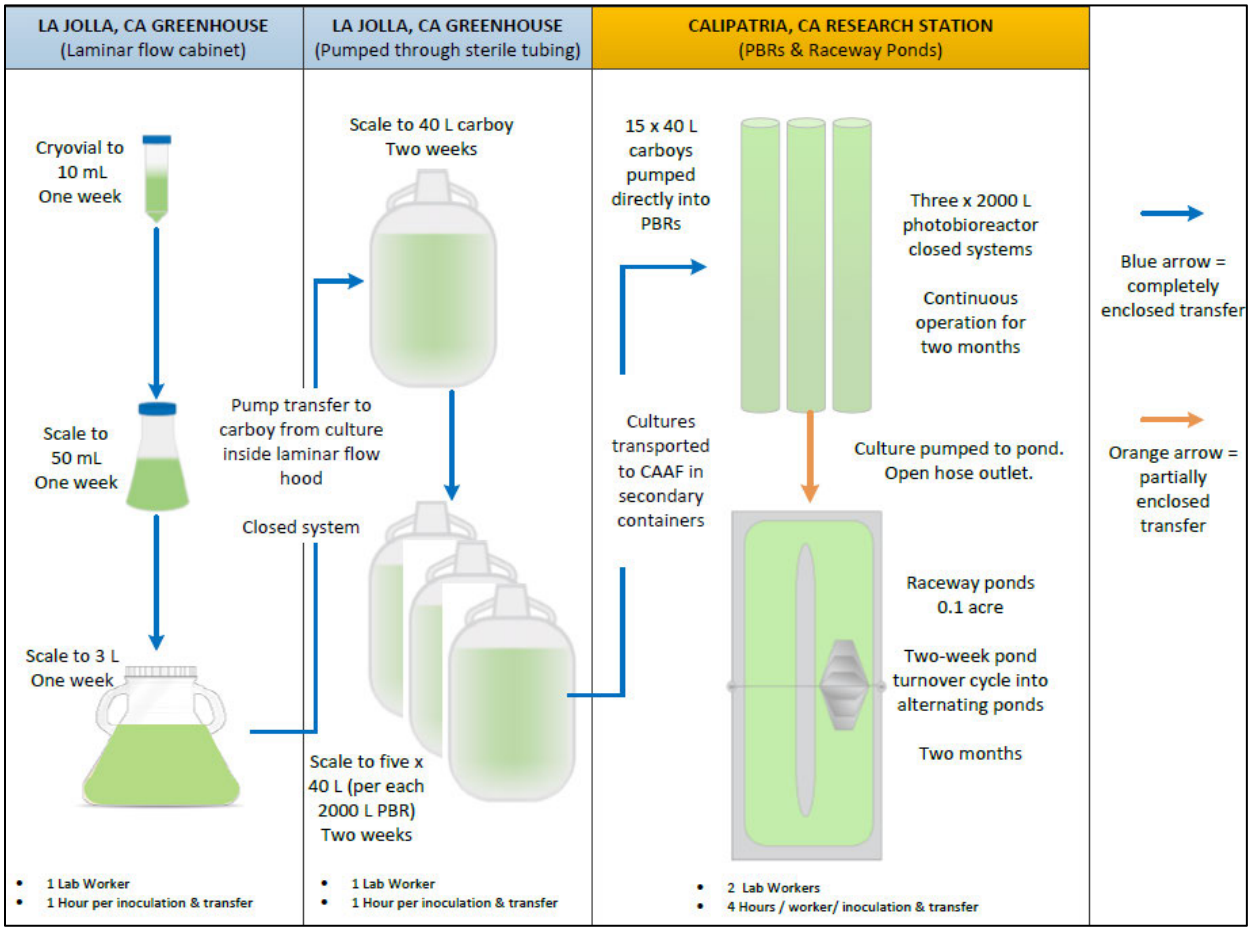
1. Production Volume

The submitters indicate that the ponds will reach a maximum density of 5 x 10^7 colony-forming units (CFU)/ml. There will be 12 batches maximum, once each week during a 3-month period (according to technical contact). Thus, EPA assumes 3 months and 12 batches as a conservative estimate. The submission states that the ponds in use will alternate between the two ponds. Based on this information, the total maximum production volume for the submission microorganism *Parachlorella* sp. STR26155 is 6.0 x 10^{16} CFU for this field trial.

2. Process Description

The subject microorganism was created within the labs at SGI. The strain is then transported to the SGI La Jolla Greenhouse (within the same research park) in sealed secondary containers. There, the cultures are maintained and scaled prior to movement to the CAAF. Shipment of the subject microorganism will be made in clearly-labelled, sealed containers of approximately one to three liters. These will be further contained in secondary spill-proof containers and transported with enough bleach to neutralize the cultures in the case of a catastrophic failure. The technical contact submitted 'SGI Algal biofuels culture scale up process and associated worker exposure' which indicated that the strain will be scaled up to five 40 L carboy then transferred to the Calipatria Research Station (Figure 9).

Figure 9. SGI Algal biofuels culture scale-up process and associated worker exposure.



As depicted in Figure 9, seed stocks will be maintained in a dedicated grow room and transferred only between sealed containers during the scaling process. Once at least 100 L of seed has grown to a density of at least 1.0 g/L, the seed stock will be utilized to inoculate the 2,000 L and 4,000 L PBRs at a density of approximately 0.1 g/L. Once the PBRs reach a density of at least 1.0 g/L, they will inoculate one of the 0.1-acre ponds at a target operational starting density of 0.1 g/L. These ponds will then run for one week each. At the end of a week of growth, the ponds will be deactivated and disposed.

PBRs and ponds have secondary containment in the form of a 24-inch berm that is lined with a mesh reinforced, puncture resistant, UV-resistant material. The berm has an effective footprint of 1 acre and can hold the approximately 5x the capacity of the two 0.1-acre L ponds plus all PBRs, in the highly unlikely scenario of complete primary containment failure.

The submitter will regularly sample multiple sample types from a variety of sites (e.g., bioaerosols, trap ponds, CAAF production ponds, local environmental sampling) to provide data on the potential release of the engineered alga from the experimental ponds. The submitter will conduct active monitoring for one week prior to the start of open engineered alga cultivation, during the entire course of the experiment, and for 2 weeks following termination of the engineered alga ponds. During this active monitoring period, one type of endpoint will be the five 350 L “algae-trap” ponds established to help assess the dispersion capability of the subject organism. Additionally, the submitter will sample regularly from all other ponds on site that are in active use and assay for the presence and abundance of the subject strain. Lastly, regular bioaerosol samples will be collected and similarly assayed for the presence and abundance of the subject strain. Both during the active monitoring, and for one year following first inoculation, the submitter will continue to carry out passive monitoring consisting of monthly sampling from established environmental stations.

Samples will be collected daily for the CAAF Lab to perform growth measurements. Briefly, these measurements will include optical density (OD730), ash-free dry weight (AFDW), photosynthetic efficiency (PAM), total organic carbon (TOC), fatty acid methyl ester composition (FAME), microscopic analysis and metagenomic analyses. Excess samples will be disposed of in 0.5% sodium hypochlorite. The culture will be inoculated with media containing nitrogen, phosphorus, and trace minerals.

At the end of each experiment, the ponds will be deactivated-in-place with at least 4 mL/L of a 12.5% sodium hypochlorite solution before disposal in the site’s evaporation pond.

Clean-in-place procedures are utilized for cleaning ponds at the CAAF site. At the conclusion of an experiment, ponds are scrubbed along the sides with brushes to remove any films that may have formed over the course of an experiment. Then, ponds are dosed with 4 mL/L of 12.5% sodium hypochlorite and thoroughly mixed with the in-pond paddlewheels. After at least one hour, and after complete mixing, the ponds are then pumped directly to the on-site evaporative disposal pond via a dedicated line.

To ensure that the subject microorganism is completely removed from the test site after the experiment has been completed, all liquid biomass will be treated with 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal. This dose is 12.5-fold greater than the experimentally determined effective dose for killing both recipient and subject strains. Scale up vessels, including Fernbach flasks and carboys, will be treated with bleach to neutralize the microorganism before dumping down the drain to the evaporative pond. Carboys will be cleaned and autoclaved for reuse. 0.1-acre ponds will be deactivated in place with bleach before disposal into the evaporative pond. Samples that have been collected from the site will be neutralized by treatment with 4 mL/L of 12.5% sodium hypochlorite for a minimum of one hour before disposal.

3. Worker Exposure

The occupational exposure to the algal submission strain for the proposed field test has been estimated by Hollinshead (2019). There will be a total of up to 20 workers.

The worker estimates provided in the submission are given in Table 6.

Table 6. Worker estimates by activity.

Worker Activity	PPE	# of Workers Exposed	Maximum Duration (hr/day)	Maximum Duration (day/yr)
Scale-up of cultures	Proper PPE : gloves, safety glasses, long pants, and steel-toed shoes	3-4	4	52
Inoculation of ponds		3-4	4	52
Sampling of ponds		3-4	1	365
Sample processing (lab)		3-4	2	365
Experimental termination		3-4	4	52

The submission indicates that proper PPE, including gloves, safety glasses, long pants, and steel-toed shoes will be used as required by SGI regulations.

INHALATION EXPOSURE (bioaerosols):

1) From sampling near paddlewheels: 60 to 411 CFU/day, up to 4 workers (Table 6.)
2) From inoculation and scale-up, PBR sampling, sample processing, and experimental termination: negligible

DERMAL EXPOSURE:

1) From laboratory work, scale-up, and daily sample processing: 2.0×10^7 to 5.5×10^7 CFU/day, up to 20 workers/site, up to 98 days/yr

This dermal exposure estimate assumes spillage onto unprotected skin. However, PPE (i.e., gloves) will be worn.

4. Environmental Releases

WATER: Not expected
(The submission states that the CAAF is a zero-discharge site for wastewater. Post inactivation, PBRs are pumped to an onsite evaporation pond. Evaporated biomass is subsequently sent to landfill.)

AIR:

1) From bioaerosol emissions: 7.1×10^5 CFU/day over 84 days/yr; 6.0×10^7 CFU/yr
2) From fugitive emissions during sampling: negligible

LANDFILL:

1) From PBR cleaning: negligible
2) From unused PBR biomass termination: negligible
3) From pond/equipment cleaning: 1.0×10^7 CFU/day over 12 days/yr; 1.2×10^8 CFU/yr
4) From pond termination: 4.9×10^8 CFU/day over 12 days/yr; 5.9×10^9 CFU/yr

INCINERATION: Not expected
(The submission indicates that waste will be disposed to the evaporation pond and subsequently

landfilled.)

5. Consumer, General Population, and Environmental Exposure

The exposures to consumers, the general population, and to the environment were estimated by Lynch (2019).

a. Consumer Exposure

The algal submission strain is not intended for use in consumer products. Therefore, exposure to consumers is not expected.

b. General Population Exposure

There are releases of the algal submission strain to air and landfill from processing/use. However, landfill disposal regulations (state and federal), landfill design and management practices and low emissions to air are expected to mitigate the exposures to negligible levels (Lynch, 2019).

Exposure to Releases from Processing

1. Inhalation Exposure from Bioaerosol Fugitive Emissions

Using the estimated maximum release of 6.0×10^7 CFU/yr the concentration in ambient air 100 meters downwind would be much less than 1 CFU/m^3. Thus, exposures are expected to be negligible (<1 CFU/yr).

It should be noted that examination of satellite imagery of the processing/use site indicated the closest residences to be approximately 1.5 miles from the site.

2. Drinking Water Exposure

No drinking water exposure to the submission microorganism is expected from the proposed open pond field tests. According to the submission, the CAAF is a zero-release facility where no releases to water will occur. After inactivation 4 ml/L of 12.5% sodium hypochlorite (bleach) for at least one hour, all the pond liquid and liquid created by cleaning of PBRs and other equipment is sent to an on-site evaporative pond. After evaporation, the residues in the pond are sent to a landfill.

XVII. INTEGRATED RISK ASSESSMENT

Although there are some uncertainties regarding the ability of the submission strain *Parachlorella* sp. STR26155 to be dispersed from the testing site through aerosols and the subsequent survival of the alga in the environmental media into which it may be disseminated, the proposed small-scale field test does not appear to pose unreasonable risks to human health or the environment.

The recipient alga, *Parachlorella* sp. STR00012*,* is not known to be pathogenic to humans. Although there have been three cases of infection in humans caused by unspecified species of the closely-related *Chlorella,* chlorellosis in humans is extremely rare as *Chlorella* is omnipresent in the environment in both fresh and marine waters, and in soils so humans are frequently exposed to the alga. Also, these three infections were a result of open wounds being exposed to contaminated waters.

The introduction of TurboGFP in the submission strain does not pose any increased concern for human pathogenicity. The family of GFPs and GFP-like proteins that have been isolated from numerous organisms have been utilized as reporter proteins in many microbial, plant, and animal assays with minimal impact to their phenotype. The introduced genetic material does not pose increased risk of pathogenicity to susceptible subpopulations, i.e., immunosuppressed individuals. Even in extremely rare circumstances of exposure of open wounds in severely immunocompromised individuals to contaminated waters, the submission strain is not expected to pose any concerns that are not already associated with the wild type recipient strain.

There is no concern for toxicity to humans because no species of *Parachlorella* is known to produce phycotoxins. The closely-related *Chlorella* spp. are commonly used as human dietary supplements, and thus, do not pose toxicity concerns. The introduced genetic material does not pose any toxicity concerns even to susceptible subpopulations.

There is low concern for allergenicity in workers and to the general population from exposures to the genetically modified algae during this field test. The frequent detection of *Chlorella* in bioaerosols in the environment implies that humans are routinely exposed to *Chlorella* (and likely *Parachlorella*) by the respiratory route. Adverse allergic reactions to algae in bioaerosols is thought to be less of a concern that other airborne environmental antigens such as bacteria, fungi, and pollen spores.

However, *Chlorella,* and thus potentially *Parachlorella*, may cause sensitization in the susceptible subpopulation of atopic individuals, i.e., those with a genetic predisposition toward developing hypersensitivity reactions upon exposure to environmental antigens. In one study, *Chlorella* was suspected of being a weak allergen, however, no studies have established a definite causal role for *Chlorella* in human respiratory allergies. The algal cells are expected to occur in bioaerosols generated through the turbulence of the paddle wheels in the raceway miniponds or through wind action. Since *Parachlorella* cells are very small and growth is unicellular, the alga is known to be easily transported in the air. It is unlikely that atopic individuals would choose to work with algae at the SGI facility given their predisposition for respiratory hypersensitivity reactions with exposure to environmental antigens. However, if any workers are atopic, or non-atopic individuals were to developed allergy symptoms, the use of respirators would mitigate allergenic responses.

There is no increased concern for the respiratory exposure of the submission microorganism *Parachlorella* sp. STR26155 compared to that of the recipient alga. The TurboGFP protein is intracellular, thus, there is no direct respiratory exposure to the protein. There is low concern for allergenicity through respiratory exposure of the submission strain *Parachlorella* sp. STR26155 to the general population as inhalation exposures resulting from this small-scale field test are expected to be quite low.

Likewise, environmental hazards resulting from this small-scale field test are expected to be low. Although there have been a few cases of chlorellosis in several animals, infection of non-human mammals by *Chlorella* is very rare as there are a limited number of cases even though *Chlorella* is one of the most prevalent algae in the environment in marine and fresh waters and in soils. There is low concern for potential toxicity of *Parachlorella* to animals since no members of the genus are known to produce phycotoxins. There is no literature suggesting that *Chlorella* or *Parachlorella* have any adverse effects on terrestrial or aquatic plants.

As previously discussed, *Parachlorella* sp. is not known to produce any toxins that might be harmful to humans, animals, or plants. Although resistance genes to the antibiotics chloramphenicol and zeocin were used in the development of the subject strain STR26155, they are not present in this final submission strain. The genetic modifications made to the recipient microorganism are not expected to introduce any other phenotypic change in the recipient microorganism and does not impart or enhance

any harmful traits beyond what may be present in the recipient strain. The proposed field test of *Parachlorella* sp. STR26155 poses low concern for humans and the environment as the genetic modification of introducing TurboGFP also poses low hazards.

As discussed by Henley et al. (2013) in their analysis of the risks posed by commercial scale production of GE algae, there are several scenarios that should be considered if a GE alga is grown outdoors, and hence, is likely to be disseminated to other environments. It is possible that a GE algal strain would die off in a new environment, and thus, there would be low risk. However, even the scenario of low-level survival of a GE strain in the environment does not in and of itself pose risk. If there is low-level survival of the GE strain, then the selective advantage imparted to indigenous populations through horizontal gene transfer must be considered. If the horizontally transferred trait imparts a nonsignificant selective advantage to indigenous species, then there is low risk. If the horizontally transferred trait imparts a significant selective advantage to indigenous species, then there could be some risk. A scenario of high risk may be considered when the GE algae dominates a new environment and causes hazardous algal blooms or ecosystem-disruptive algae blooms (EDABs), but as previously discussed, *Parachlorella* do not produce phycotoxins and thus do not cause HABs.

The potential for horizontal gene transfer of the TurboGFP gene to other algae in the environment is thought to be low as *Parachlorella* is not known to readily exchange genetic material horizontally. Very little is known about horizontal gene transfer in eukaryotic algae as it has not been observed.

Thus, even though the submission strain may be dispersed into other environments as is the case with any algae grown outdoors, there is low risk associated with the dispersal to and survival in other environments into which it may be disseminated.

CONCLUSIONS

The proposed field test with the subject microorganism *Parachlorella* sp. STR26155 does not pose an unreasonable risk to human health or the environment. This small-scale field test is to establish baseline environmental conditions in and around the CAAF test facility, and to evaluate and confirm the sufficiency of control and monitoring equipment and techniques developed for this and other similar outdoor R&D programs. This TERA also aims to lay the foundations necessary to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. The submitters hope to gain insight into how current and yet-to-be-developed algal strains perform in industrially-relevant settings to help inform the future design of algae for biofuels technology.

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